A Chemical Genetic Approach To The Study Of Cellular Transport

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VRIJE UNIVERSITEIT

A Chemical Genetic Approach To The Study Of Cellular Transport

ACADEMISCH PROEFSCHRIFT

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de Vrije Universiteit Amsterdam,
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door

Thomas Johan Frederik Nieland

geboren te Leiden

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'.....it is hard work.....'

George W. Bush
First presidential debate
September 30, 2004

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The research described in this thesis was performed between November 1999 and November 2004 at the Department of Cell Biology and the Harvard Institute of Chemistry and Cell Biology at Harvard Medical School and the CBR Institute for Biomedical Research, Boston, Massachusetts, USA, and at the Department of Biology at the Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.

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CONTENTS

CHAPTER I

Introduction

Scope of this thesis

This thesis focuses on the use of chemical genetics to study two different aspects of membrane biology, (a) the mechanisms underlying cellular lipid transport and (b) the intersection between endocytic and exocytic traffic. The broad goals of chemical genetics are to find novel chemical tools to study biological systems and to identify novel molecular players therein. A key advantage of small molecules is their ability to rapidly and reversibly interfere with biology which qualifies them as ideal reagents to study the dynamics of complex processes (1, 2).

Cellular cholesterol homeostasis is maintained at the level of synthesis and catabolism of cholesterol, and through regulation of cellular lipid import and -export. Lipids can be acquired from the extracellular environment by *receptor-mediated endocytosis* of specialized carriers, such as low-density lipoproteins (LDL), or by dedicated surface membrane *transport proteins* (3). LDL-receptor mediated endocytosis moves LDL particles into endosomal/lysosomal compartments where cholesterol and other lipids are transferred into the cell proper, concomitantly with the degradation of the lipoprotein (4). In contrast, surface membrane lipid transporters directly transfer lipids between extracellular ligands and the cell surface bilayer independent of cellular endocytic activity. The transport mechanisms of two lipid transport proteins, Scavenger Receptor, Class B, type I (SR-BI), and ABCA1 (ATP Binding Cassette transporter A1), are investigated in the first part of this thesis. Both transporters play critical roles in lipoprotein metabolism, atherosclerosis and cardiovascular disease (5, 6).

The second part of this thesis describes a study of the mechanisms of intracellular trafficking. For a multicellular organism to function properly, it is imperative that cells coordinate their activities. Careful modulation of exocytic- and endocytic traffic allows cells to communicate with each other via controlled surface expression of membrane proteins, and via the secretion and internalization of soluble molecules. The information that these soluble molecules carry can be interpreted in autocrine, paracrine or endocrine fashion. Because many molecules need to be directed (and restricted) to their appropriate intracellular location following their cellular synthesis or acquisition from the outside world, controlling cellular trafficking also ensures the preservation of the identity of subcellular compartments. Finally, to fulfill their metabolic needs, cells use intracellular trafficking pathways to take up nutrients, and other molecules they cannot synthesize, from the outside world (3). Experiments described in the second part of this thesis aspire to characterize molecular elements that regulate both endocytosis and exocytosis. This line of research experiments is motivated by a desire to deepen our understanding of the basic machinery of cellular traffic.

Part I, Cholesterol Homeostasis

An overview

Cholesterol is indispensable for numerous cellular functions, including cell signaling, protein modification and synthesis of a variety of biologically active molecules such as steroid hormones and bile acids. In addition, cholesterol performs a structural role in cellular membranes. This puts a strict demand on the maintenance of cholesterol homeostasis, which can be accomplished by controlling cholesterol synthesis and -metabolism, and by bi-directional

cholesterol transport between cells and dedicated carriers, called lipoproteins[1], in the circulation (3).

Synthesis of cholesterol is a multi-step process carried out by a large number of enzymes that reside either in the cytosol or at the membrane of the endoplasmic reticulum. Because excess of cellular cholesterol can be toxic (e.g. (7)), cells have the capacity to reduce cholesterol levels by negative feedback mechanisms that put a brake on cholesterol synthesis, and by storing cholesterol in lipid droplets in the form of cholesteryl esters. In addition, lipid transport proteins can donate (excess) cellular cholesterol to extracellular carriers such as high-density lipoproteins (HDL). Conversely, when cholesterol levels are low, cells may respond by increasing cholesterol synthesis or acquisition of cholesterol from HDL, LDL and other lipoproteins in the circulation. This requires specialized surface LDL-receptors to internalize low-density lipoproteins into a lysosomal compartment, where cholesterol is made available to the cell upon degradation of the lipoprotein. Other membrane receptors (e.g. SR-BI) act as lipid transport proteins to transfer lipids from lipoproteins to cells without the concomitant internalization of receptors or lipoproteins (3). The role of endocytosis in lipid transport mediated by SR-BI is investigated in chapter IV of this thesis. The precursors of LDL (very low density lipoproteins or VLDL) and HDL (pre-β-HDL, see below) are assembled in the liver and mature in the circulation to the final product (6). A more detailed description of lipoprotein metabolism is beyond the scope of this thesis.

Genetic- and environmental (high fat, western-style diet; sedentary lifestyle) factors can have a severe impact on cholesterol homeostasis, potentially leading to the development of life-threatening diseases such as atherosclerosis and cardiovascular disease (CVD). Indeed, many genetic disorders have been identified that alter expression and/or activity of lipoprotein receptors, as is the case with LDL-R (in familial hypercholesterolemia) (8), lipid transporters (such as ABCA1 in Tangier Disease (9-12), ABCG5 and ABCG8 in sitosterolemia (13-15)), apolipoproteins (e.g. in familial defective apolipoprotein B-100 (16)), or enzymes involved in cholesterol synthesis or metabolism (17). A famous risk factor associated with cholesterol-related diseases is the level of HDL and LDL in the plasma. HDL is also known as *good cholesterol* because high levels of this lipoprotein inversely correlate with the chance of developing atherosclerosis and CVD. As detailed below, this has subsequently been attributed to the role of HDL in reverse cholesterol transport. In contrast, high LDL levels predispose for the development of these diseases. LDL is referred to as *bad cholesterol* because, paradoxically, its beneficial function as a cholesterol-delivery vehicle can unintentionally wreak havoc during an inflammatory response in the arterial system, which may lead to activation of specialized cells called macrophages. These cells develop the capacity to vigorously internalize cholesterol from a modified (oxidized) form of LDL, which arises when plasma LDL levels are high. Consequently, these macrophages transform into lipid-laden foam cells, marking the beginning of what could become an atherosclerotic plaque. A growing plague progressively reduces the blood flow through the vasculature, thereby depriving tissues from oxygen and nutrient (ischemia). If the plaque grows to a size that severely or completely occludes the arteries that serve the heart or the

[1] Lipoproteins are spherical assemblies of a lipid core and an outer shell composed of characteristic apolipoproteins and lipids, which differ from those within the core. Different classes of lipoproteins are recognized based on their protein components and lipid content, and hence the density of the particle. For instance, apoA-I is the most prominent apolipoprotein in HDL, and apoB the sole protein found in LDL. The location of the lipids in the lipoprotein depends on their physico-chemical properties; amphipatic phospholipids and unesterified cholesterol are found in the outer shell and triglycerides and cholesteryl esters are located in the core.

brain, or when a blood clot forms upon rupture of the plaque, a heart attack or a stroke may result. LDL levels thus positively correlated with the chance of developing arteriosclerosis and cardiovascular disease (3).

Scavenger Receptor, class B, type I (SR-BI)

SR-BI was identified as the first authentic cell surface receptor and lipid transporter for high-density lipoproteins (18). In a process called *selective uptake*[2] (19-21), SR-BI transfers lipids from extracellular HDL (18) or LDL (22, 23) to cells. Cholesteryl esters are important lipids to be transported, but other lipids (unesterified cholesterol, phospholipids, triglycerides) and hydrophobic molecules (α-tocopherol) can be transferred too (24-31). Unlike lipid uptake from LDL via the LDL-receptor (4), SR-BI mediated selective uptake does not require degradation of the lipoprotein (18) or endocytosis of the receptor-lipoprotein complex (see chapter IV of this thesis and (32)).

SR-BI is predicted to have two transmembrane domains and short intracellular N- and C-terminal tails (33). Due to heavy glycosylation, the apparent molecular mass of this 57kDa protein is about 82kDa (34). Genetic and chemical genetic analysis (chapter II of this thesis) and a functional comparison to the SR-BI family member CD36 have shown that binding of lipoproteins to the extracellular portion of SR-BI is required, but not sufficient, for selective uptake to occur (35-37). Thus, SR-BI mediated selective uptake is a two-step process, which demand a so-called productive binding of HDL to the receptor followed by the physical lipid transfer to the cell (35, 38).

True to its name, SR-BI is able to recognize a variety of molecules, such as proteins (e.g. apoA-I (39-41)), lipids (anionic phospholipids) (42, 43) and carbohydrates (lipopolysacharides [LPS] (44), advanced end-linked glycan [AGE] modified proteins (45)). A physiological relevance has been assigned for the interaction with lipoproteins, (e.g. HDL, LDL, chylomicrons (46) and see (6)), but for others, including apoptotic cells (42, 47) and hepatitis C virus (48), this remains to be established.

Reverse cholesterol transport

In murine experimental models, genetic manipulation of SR-BI expression levels has demonstrated an important role for SR-BI and HDL in reverse cholesterol transport (RCT), a process believed to offer protection against atherosclerosis and cardiovascular disease (49-56). In reverse cholesterol transport, cholesterol is moved from extra-hepatic tissues to the liver. In addition, cholesterol can also be transported to steroidogenic tissues. RCT avoids accumulation of pathological levels of cholesterol in peripheral tissues, such as arterial macrophages. The first step of reverse cholesterol transport is efflux of cellular unesterified cholesterol, potentially mediated by ABC proteins (57) (as will be further explained in a following section), to spherical HDL (α-HDL) bound to non-hepatic cells. In the circulation, HDL is subsequently remodeled by a variety of enzymes, such as lecithine cholesteryl acyl transferase (LCAT), which catalyzes the conversion of unesterified cholesterol to cholesteryl esters (58).

SR-BI mediated selective uptake of HDL cholesteryl esters and other lipids occurs predominantly in the liver and steroidogenic tissues (testis, ovaria, adrenal gland), the tissues that express the highest levels of SR-BI (59). Steroidogenic tissues hydrolyze cholesteryl esters to produce cholesterol, which is used to synthesize steroid hormones. In the liver, a portion of the

[2] Although SR-BI independent selective uptake pathways exist, this thesis will refer to selective uptake as SR-BI mediated selective uptake.

cholesteryl esters is stored in lipid droplets, secreted into the bile after conversion to bile acids, or reused for biogenesis of nascent HDL and other lipoproteins. In addition to SR-BI mediated selective uptake, a fraction of the cholesterol in HDL can be transferred to LDL in the form of cholesteryl esters by cholesteryl ester transfer protein (CETP), which then can be delivered to the liver via LDL-receptor mediated endocytosis (6, 60).

Cholesterol efflux from macrophages to HDL in the first phase of reverse cholesterol transport is presumed to be facilitated by members of the ATP-binding cassette (ABC) transporter family, of which ABCA1 (61) and, more recently, ABCG1 and ABCG4 have received the most attention (57). In this context, it is worth mentioning that SR-BI can also mediate efflux (as well as selective uptake) of unesterified cholesterol (but not cholesteryl esters) from cells to HDL, LDL and other non- lipoproteins carriers (24, 38, 62). This efflux pathway may represent either a net efflux or an exchange process at steady state, depending on the direction of the cholesterol concentration gradient between cells and lipoprotein particles (63). Although the physiological relevance of SR-BI dependent lipid efflux is largely unknown, there is speculation that SR-BI could be responsible for the release of excess cholesterol from macrophages to HDL. Consistent with this is the observation that targeted expression of SR-BI in macrophages reduces the development of atherosclerotic lesions (56, 64). However, the role of SR-BI in the macrophage appears to be complex. Indeed, in relevant mouse models the receptor promotes formation of atherosclerotic lesions in young animals, presumably by increasing lipid loading in macrophages as a result of cholesteryl ester selective uptake, but it counteracts plague development later in life, perhaps by facilitating efflux of cellular cholesterol (65). These results imply a need to balance the direction of lipid transport by SR-BI.

Physiological importance of SR-BI

By mediating selective uptake, SR-BI is a critical component in moving excess of lipids from the periphery to the liver and steroidogenic tissues, where there is a use for it, thereby avoiding atherosclerosis and CVD. In addition, as a consequence of its role in facilitating selective uptake, SR-BI is crucial in controlling both metabolism and structure of HDL (18, 60). Maintenance of the HDL pool is required to ensure that sufficient lipid carriers are available for reverse cholesterol transport, and to carry out additional anti-atherogenic activities. For instance, HDL reduces pro-inflammatory LDL oxidation and promotes the release of anti-inflammatory nitric oxide (NO). Indeed, HDL binds molecules with anti-oxidant properties such as the enzyme paraoxonase and α-tocopherol, the biologically active form of vitamin E (28, 30). Paraoxonase reduces the lipid-peroxidation level of pro-atherogenic modified LDL lipoproteins, whose uptake by macrophages otherwise promotes formation of atherosclerotic lesions (66, 67). Binding of HDL to SR-BI leads to the production of the anti-inflammatory signaling molecule NO by eNOS (endothelial Nitric Oxide Synthase) (68). NO stimulates relaxation of the endothelial wall, facilitating circulation of the blood. Nitric oxide is also known to reduce the risk of arterial plaque formation by modulating leukocyte and platelet adherence to the endothelium (69). The molecular mechanisms of SR-BI and HDL mediated eNOS activation are somewhat controversial. SR-BI mediated lipid selective uptake is believed to stimulate localization of eNOS to caveolae (70). Here, eNOS activity is induced by HDL associated lysophospholids (71) and/or estradiol (72) via stimulation of signaling cascades that rely on protein- and lipid kinases (73), synthesis of ceramide lipids (74) and/or mobilization of intracellular Ca^{2+} (69). Further anti-atherogenic activities ascribed to SR-BI are based on the increase in expression of genes involved in adhesion and transendothelial migration of monocytes in SR-BI null mice, which is

speculated to be caused by alterations in cholesterol homeostasis. This has led to the proposal that the receptor may control inflammatory responses in aortic vessels (75).

In addition to the effects on arteriosclerosis and cardiovascular disease**,** the physiological significance of SR-BI is further highlighted in female fertility (49) and reticulocyte maturation (76), which are both compromised in SR-BI deficient mice. This may be related to changes in lipoprotein metabolism and, consequently, in cellular cholesterol levels (76, 77). Contrary to expectation, infertility cannot be explained by insufficient production of steroid hormones in the ovaries, such as progesterone, because this appears to be adequate (49).

The cellular mechanism of SR-BI mediated selective uptake

Although a wealth of information is available on the physiological effect of SR-BI in various disease models and on lipoprotein metabolism, and on the details of lipoprotein binding, a physical and biochemical understanding of the lipid transport mechanism is limited.

It is now generally accepted that binding of lipoproteins to SR-BI is necessary for selective uptake to occur. Williams, Phillips and colleagues hypothesized that SR-BI forms a *channel* through which lipids are transported from the lipoprotein into the cell (78). An alternative model by Krieger and co-workers proposes that lipid transfer is dependent on *hemi-fusion* of lipids in the outer shell of HDL and the outer leaflet of the plasma membrane, allowing for movement of core cholesteryl-esters into the surface membrane (35). There is speculation that oligomerization of SR-BI is required for selective uptake to occur (79).

Mice deficient in SR-BI show changes in lipoprotein profiles, most notably an increase in the size and lipid- and protein composition of HDL particles (50). This has led to the idea that transfer of lipids from HDL reduces the physical size of the lipoprotein, which is considered to be sufficient to reduce the apparent affinity of SR-BI for HDL, allowing for the release of the lipoprotein (38, 80-83). This liberates the receptor for another round of selective uptake with a newly incoming lipoprotein.

Genetic- and chemical genetic approaches have been applied to understand the relationship between lipoprotein binding, lipid transfer and other activities of SR-BI. These studies have demonstrated that lipoprotein binding is required for lipid transport to occur, and that both processes are tightly coupled (35-37). Differences exist between HDL and LDL with regard to binding to SR-BI (84). Suggestions that SR-BI needs to form a special membrane microenvironment at the surface to facilitate selective uptake (85) have recently been discredited (86). Furthermore, the mechanisms of SR-BI mediated selective uptake and efflux may share similarities, but they do not need to be identical. Indeed, cholesterol efflux is less sensitive to certain chemical inhibitors targeting SR-BI activity (37), and genetic mutations in SR-BI reduce cholesterol efflux more pronounced than selective uptake (79). Finally, the exploitation of yet a different set of SR-BI mutants has indicated that cholesterol efflux to HDL may differ from efflux to non-lipoprotein acceptors (86).

It is clear from *in vitro* reconstitution experiments in liposomes that SR-BI supports selective cholesteryl-ether (a non-hydrolyzable analogue of cholesterol-esters) uptake without the need for accessory proteins or specialized lipid environments (87). However, this does not preclude that other factors may modulate the efficiency of SR-BI dependent lipid transport *in vivo*. Indeed, hepatic lipase (88) and lipoprotein lipase (89) are reported to facilitate cellular selective uptake, but this seems to be independent of SR-BI (although for a different view on hepatic lipase see (90)).

Cellular location of SR-BI mediated selective uptake

Currently, it is uncertain whether (cholesteryl ester) selective uptake from HDL requires endocytosis of the lipoprotein. Selective uptake was originally assumed to be a cell surface event, without the need for internalization or degradation of the receptor or the lipoprotein (as observed for lipid uptake from LDL via the LDL-receptor pathway (4)). This statement is based predominantly on electron microscopic- and biochemical analysis showing a lack of internalized HDL particles in a variety of primary and immortalized cell lines, and on the observation that selective uptake is accompanied by very little degradation of HDL (18-20, 91-93). By depleting cells of ATP, it was recently shown that endocytosis of HDL is not needed for SR-BI mediated uptake of fluorescent cholesterol analogues from HDL. However, it was suggested that this may be different for the transfer of esterified cholesterol (32). Finally, SR-BII, an alternative splice form of SR-BI that differs only in the short C-terminal intracellular segment (94), efficiently internalizes HDL particles into an intracellular environment that appears not to be optimal equipped for lipid transport (95). Together, these data suggest that SR-BI mediated selective uptake occurs at the cell surface.

In sharp contrast to this 'cell surface model', Silver and co-workers have proposed that SR-BI mediated selective uptake occurs intracellularly (96). This is supported primarily by their observation that a (small amount) of fluorescently labeled HDL is endocytosed in primary hepatocytes and cell lines expressing recombinant SR-BI (96), and on experiments which show that a reduction in HDL recycling is associated with diminished selective uptake in *ob/ob* mice (97). Indeed, it had been shown before that HDL particles can be internalized by a variety of cells, but these studies did not examine a possible involvement of SR-BI (98-105), and SR-BI-independent endocytic pathways for HDL are recognized too (e.g. via the cubulin-megalin receptor pathway (106-108)). In this thesis, a functional link is investigated between SR-BI mediated selective uptake of cholesteryl esters and endocytosis of lipoproteins. The results presented in chapter IV suggest that selective uptake from HDL or LDL carried out by SR-BI occurs at the cell surface and does not require endocytosis of the lipoproteins.

Intracellular components of SR-BI mediated lipid transport

Cholesteryl esters transferred to the cell via SR-BI mediated selective uptake first enter a reversible membrane pool, meaning that the lipids can be shuttled back to HDL (19-21), and subsequently move into an irreversible pool (109, 110). One study has proposed that the lipids are irreversibly incorporated in a soluble, non-membranous, cytosolic complex consisting of the cholesterol-chaperone caveolin, cyclophilin A, cyclophilin 40 and Annexin II before entering an internal membrane compartment(s) of unknown identity (111). In favor of a role for caveolin/caveolae in lipid transport is the observation that SR-BI and caveolin-1 are found by immunofluorescence and biochemistry in the same subcellular fraction (34). However, a confounding fact is cells that do not express caveolin support efficient SR-BI mediated lipid transport, suggesting that the caveolin protein is dispensable (112, 113). This is substantiated by recent high-resolution electron microscopy analysis showing that SR-BI and caveolin are juxtaposed, but not colocalized, in specialized membrane structures (91). Still other reports claim that caveolin is a negative regulator of selective uptake by SR-BI (114). Clearly, a more thorough study is required to fully comprehend the role of caveolin in SR-BI mediated lipid transport.

It is not unlikely that the mechanistic details of SR-BI activity depend on the cellular context, and this may at least in part account for the apparent contradictory results on the role of

caveolin. An example is offered by studies of PDZKI knock-out mice. PDZK1 is a scaffold protein that post-translationally stabilizes hepatic SR-BI (115). It binds the C-terminal intracellular domain of the receptor (116). In PDZKI deficient mice, hepatic SR-BI levels are undetectable, and HDL levels and -metabolism are affected consequently in a manner similar to animals that do not express SR-BI. Importantly, the effect of PDZK1 deficiency on SR-BI expression is a cell-type specific phenomenon, because the steady-state level of SR-BI protein is only decreased in the liver and not in steroidogenic tissues, the other major site of SR-BI expression (115).

Events downstream of the initial selective uptake step have been probed with chemical reagents. These studies revealed for instance that HDL and LDL-born cholesteryl esters are hydrolysed by neutral hydrolases in a compartment of unknown identity (117).

ABCA1

ABCA1 is a member of the family of ATP Binding Cassette (ABC) transporters, which is characterized by the ability to transport small molecules (xenotoxins, endogenous metabolites, lipids) across cellular membranes in an ATP dependent fashion (118). ABCA1 is a full transporter, meaning that it consists of twelve transmembrane helices, two large extracellular loops and two intracellular Walker A/B motifs responsible for ATP-hydrolysis (12, 119, 120). Several ABC proteins, including ABCA1 (9-12), ABCA7 (121-123), ABCG5 and ABCG8 (13-15), ABCG1 and ABCG4 (57) and ABCB11 (124) have been implicated in cholesterol homeostasis and/or cellular lipid transfer.

The physiological importance of ABCA1 became apparent in Tangier disease patients, who suffer from neuropathies, splenomegaly, hepatomegaly, ocular abnormalities, hypocholesteremia and cardiovascular disease (125). Similar to ABCA1 null mice (126-128), these patients accumulate cholesterol in peripheral macrophages in many of the affected organs and they have almost undetectable levels of serum HDL (9-12). The latter is the opposite finding of SR-BI deficient mice, which display an increase in the size of HDL, and a change in lipid and protein composition of this lipoprotein (50).

The absence of HDL in ABCA1 deficient individuals is consistent with the essential role of this lipid transporter in the early steps of hepatic HDL biogenesis in the liver, which synthesizes and secretes apoA-I (the precursor to HDL), and consequently in reverse cholesterol transport. At the cell surface (but see below for alternative views), ABCA1 catalyzes with low efficiency the efflux of phospholipids and cholesterol to lipid-free or lipid-poor apoA-I (12, 129-131). Consensus is emerging that apoA-I is required to bind to ABCA1 (120, 132) *and* the surrounding membrane (133) to facilitate lipid transport. It is speculated that ABCA1 needs to rearrange the membrane into a special microenvironment for apoA-I to bind (133-136). The lipidated products, called pre-β-HDL, arising from ABCA1 mediated efflux are further remodeled in the circulation into mature, spherical α-HDL particles (137-140). These particles serve as acceptors for cholesterol efflux by peripheral tissues and transport excess of lipid to SR-BI expressing tissues for selective uptake.

While SR-BI and ABCA1 share the ability to transfer lipids from cells to extracellular carriers, they have mutually exclusive preferences for lipid acceptors. Thus, SR-BI mediates cholesterol efflux to spherical HDL, but not to apoA-I, and the opposite is true for ABCA1 (12, 24, 38, 62, 129-131, 141). SR-BI binds HDL on the apoA-I moiety (41), but the structural or conformational determinants on apoA-I that are crucial for interaction differ from those

necessary for productive interactions with ABCA1 (38, 39, 142). Another difference is that unlike SR-BI, ABCA1 cannot accommodate cellular lipid import. Notwithstanding these differences, the fact that both SR-BI and ABCA1 transport lipids between cells and (different forms of) lipoproteins has catalyzed the research outlined in Chapter III of this thesis, where the use of small molecule inhibitors has led to the proposal that SR-BI and ABCA1 may share similar mechanisms of lipid transport.

Similar to SR-BI, the location of ABCA1 mediated lipid efflux is an unresolved issue. Focus has been aimed at two different, not necessarily exclusive, subcellular locations, being the cell surface (12, 120, 133, 143-146), and the endo-lysosomal system accessed by apoA-I via ABCA1-mediated endocytosis (143, 147-150). A third model proposes that at least part of the newly synthesized apoA-I is lipidated intracellularly in the liver by ABCA1 independent pathways before being secreted into the extracellular space (151, 152).

In addition to its role in HDL biogenesis, ABCA1 is thought to facilitate transport of lipids in the initial phase of reverse cholesterol transport, from macrophages in the arterial wall to HDL (9-11, 128). However, because of the very low levels of free apoA-I in the circulation (153), and the fact that ABCA1 is unable to transfer lipids to mature HDL (141), this has been a controversial scenario. Indeed, targeted expression of ABCA1 in macrophages appears to have only a minor impact on the size of the HDL pool in the circulation (154). An alternative proposal for the atheroprotective function of ABCA1 in peripheral macrophages is to control the recruitment of monocytes into the vascular wall (155, 156). One intriguing possibility is that the newly discovered ABCG1 and ABCG4 proteins, and not ABCA1, may be responsible for removal of excess lipids to spherical HDL particles in peripheral tissues (57).

Part II: Intracellular vesicular trafficking

Cellular communication, maintenance of cellular compartmentalization and transport of nutrients all require delivery of molecules to the appropriate addresses. The cell has developed different trafficking pathways that operate separately, sequentially or in parallel to ensure that traffic jams do not occur and faulty delivery is avoided. The prevalent route of trafficking depends on vesicles, but (poorly characterized) vesiculo-tubule based pathways are recognized too (157). Some of these sorting routes are cell-type specific, such as the basolateral sorting mechanisms along the exocytic trajectory of polarized cells (158).

Vesicular trafficking requires sequestration of select cargo into designated coated vesicles. Nascent coated vesicles bud from the membrane, after which the coat is shed and the naked vesicle moves to its specific destination, sometimes directed by actin- or microtubule tracts. Fusion with the acceptor membrane releases the vesicular cargo into the correct subcellular compartment (157).

Coated vesicle-based trafficking

Transport pathways can be discriminated based on whether they rely on coats to form vesicles, and if so, what class of coat proteins is employed. Four different, well-studied, coated vesicle species exist: clathrin-, COPI-, COPII- and caveolin-based vesicles (157).

The basic mechanistic steps of these coat-operated traffic routes are similar (coat formation, vesicle budding and detachment, coat release, fusion with acceptor membrane), but the molecular details differ. Cargo (soluble and membrane proteins, lipids) is selected by specific cargo receptor proteins, often based on recognition of specific molecular tags (peptide sequences,

carboydrate groups) presented by the cargo. The cargo receptors physically tether their load to coat proteins in the growing coated vesicle, sometimes at designated subcellular locations (e.g. exit sites at the endoplasmic reticulum [ER] (159)). Coat proteins nucleate the formation of membrane vesicles from a planar membrane by recruitment of other coat components. The unique combination of coat protein and cargo receptors defines the nature of the transport pathway and its destination (157).

Clathrin coated vesicles are employed in endocytosis and exocytosis to transport soluble and transmembrane proteins, of which a subset collects cargo from the extracellular milieu. Endocytosed material is either recycled back to the surface or targeted to the endo-lysomal system. Clathrin vesicles are also used at the trans-golgi network (TGN) to sort exocytic cargo to the endo-lysosomal compartment or to the basolateral cell surface (157, 158). Recent advances in single molecule tracking by fluorescence microscopy have for the first time visualized the early events in clathrin-mediated endocytosis (160).

In the exocytic trajectory, COPII vesicles bud from the ER and shuttle newly synthesized proteins to the Golgi-apparatus. COPI based vesicles move material in the opposite direction, at least in part to retrieve mistargeted proteins from Golgi back to the ER. In addition, COPI vesicles may also guide forward transport between the Golgi cisternae (157) (an alternative view is that intra-Golgi movement does not require vesicle transport, but relies on maturation of the different Golgi cisternae (161)) Some toxins, such as cholera toxin, hijack the COPI-retrograde route to gain access to the ER from the Golgi apparatus after they enter the cell. Recently, a chemical genetic study has shown that cholera toxin still can access the ER when the Golgi-apparatus is chemically destroyed, demonstrating the existence of alternative retrograde trafficking routes (162).

In addition to cargo receptors, a large variety of other proteins are specifically associated with the different coated vesicle species. They fulfill important roles in recruitment of the ligands, coat formation, detachment of the coated vesicle from the membrane and the final step of uncoating. Coats are strictly required for vesicle formation. Upon detachment of the vesicle from the membrane, coat proteins are released as individual subunits and recycled for a new round of vesicle formation (157). At least a subset of the resulting naked vesicles is attached by motor proteins to cellular networks of actin (for short range transport) or microtubules (for trafficking over long distances) for transport through the cytosol to the correct destination (163, 164). Different classes of vesicles utilize distinct motor proteins. At the acceptor site, docking proteins, including SNAREs, catalyze fusion of the vesicle with the acceptor membrane, allowing release of the vesicular content into the appropriate subcellular compartment (157).

The regulation of intracellular trafficking is not limited to protein-protein interactions. Important roles are set aside for lipids. For instance, the local synthesis and concentration of phospoinositol-4,5-bisphosphate is required to dock cargo receptors for clathrin-based vesicles at the cell surface (157). Changes in the levels of membrane cholesterol are also reported to influence trafficking (165), once again underscoring the importance of maintaining cellular cholesterol homeostasis.

Lipid rafts and caveolae

Coat structures named caveolae, made of the coat protein (and cholesterol chaperone) caveolin, have been suspected for a long time to guide cellular trafficking, but few cargo proteins have been identified. There is evidence that caveolae guide transcytosis of albumin in endothelial cells (e.g. (166)) and certain viruses may use this pathway to enter the cell (167-170). In addition

to their role in trafficking, caveolae are also thought to function as signaling scaffolds (171), for instance as described before for the case of eNOS signaling.

Cholesterol and sphingholipids can be tightly packed in lipid rafts in the membrane. Although somewhat controversial, rafts are proposed to mediate apical sorting from the TGN to the cell surface (172) and endocytosis of a particular class of post-translationally modified proteins tagged with either glycosylphosphatidylinositol (GPI), palmitoyl, dual acyl chains or cholesterol (173). Similar to caveolae, rafts are proposed to function as platforms for intracellular signaling, by limiting the lateral diffusion of membrane-associated receptor proteins (174).

A chemical link between the exocytic- and the endocytic pathway

Coated-vesicle dependent exocytosis shares many similarities with coat-dependent endocytic transport. To further characterize these similarities, small molecule inhibitors of exocytosis (175, 176) were used to search for molecules that regulate both endocytic- and exocytic traffic (Chapter V of this thesis). This has led to the discovery of a novel class of inhibitors that block intracellular transport by preventing Vacuolar-ATPase mediated proton translocation into the endocytic compartment, and possibly the Golgi (177). Indeed, precise regulation of intracellular pH is a prerequisite for proper intracellular transport as detailed in the following section.

The role of pH homeostasis in membrane traffic

The maintenance of the intracellular pH is a key requirement for optimal intracellular traffic, as well as many other biological processes, including kidney function and bone resorption. An acidic pH in endosomes is required for dissociation of cargo from receptor, which allows for recycling of receptors to the cell surface. Similarly, pH regulation in the Golgi compartment is needed for proper sorting of molecules to the surface, to lysosomes, and for secretion. The proton pumping activity of Vacuolar proton-translocating ATPases (V-ATPases) ensures proper pH homeostasis in these compartments. V-ATPases also regulate proton release from the plasma membrane, thereby affecting the cytoplasmic acid balance (178). Although the significance remains to be established, it is noteworthy that V-ATPases have been found to associate with clathrin-coated vesicles (179, 180).

Vacuolar ATPases are composed of two multi-subunit sectors, named V_o and V_1. ATP hydrolysis by the peripheral V_1 domain is required for the membrane integral V_o domain to translocate protons over the membrane against their concentration gradient (178). Not surprisingly, small molecule inhibitors for the V_o complex abolish many V-ATPase dependent processes, such as surface receptor recycling (177, 181) and transport through the Golgi and Trans-Golgi network (177, 182-184). Although speculative, inefficient exocytosis under alkaline conditions has been attributed to the inability of transport vesicles to fuse with the acceptor compartment during Golgi-transit (182).

Part III: chemical genetics

The concept of the chemical genetic approach is to screen diverse libraries of chemical compounds for their ability to interfere with biology on a molecular, cellular or organismal level. Chemical genetics aspires to find new tools to dissect biology and to use these chemicals in the search for novel molecular components that operate in the process under scrutiny. An important consideration for the choice of small molecules is that they are ideally suited to study the

dynamics of complex processes, due to the fact that in many cases they act within minutes, and their effects are often reversible. While their value is beyond doubt, classic genetic and RNA-interference based approaches to study the role of a particular gene (product) may induce (unwanted) cellular responses to the introduction or deletion of the foreign gene (product). It is therefore not inconceivable that secondary downstream effects, rather than the biology of the gene of interest, end up being investigated. This can be avoided with the use of fast acting small molecules (1, 2).

Historically, small molecules have proven to be invaluable to the study of biology, in combination with biochemical, cell biological and physiological experimentation. An additional advantage of small molecule screens is the possibility of discovering novel medicinal drugs. One of the most famous examples is the fortuitous discovery of the antibiotic penicillin from *Streptomyces sp*. by Alexander Fleming in 1929 (185). Cyclosporin A and FK506 helped elucidate the mechanisms for T-cell activation and graft-versus-host disease (186). The natural product taxol was found to posses anti-tumor activity and is a used to study microtubule polymerization (187). Histone deacetylase inhibitors are useful reagents to dissect gene regulation, chromatin and microtubule dynamics (188) and aging (189), and they are under consideration for treatment of neurodegenerative diseases (190-192). Brefeldin A (193), illimaquinone (194) and macrolide antibiotics (195) have been exploited to probe the basic mechanisms of cellular trafficking pathways and cellular entry of pathogens. Many of these chemicals were isolated from natural sources such as sponges, fungi or plants based on a particular pharmacological activity. This strategy was partly born out of necessity, because the synthesis of large collections of chemicals was at the time not feasible or not available, at least for academia (196). While the use of (focused) synthetic libraries is the most widespread today, natural- and natural-like products have regained popularity, partly from the thinking that nature, over the course of evolution, could have already invented and optimized by trial-and-error small molecules with potential experimental value (197, 198).

Reverse chemical genetics

Reverse chemical genetics encompasses the development of high-throughput chemical screens focused on a designated molecular target, protein-protein interactions or enzymatic activity, considered to be important for a particular biological or pathological process. In analogy with *reverse genetics*, chemicals are selected to specifically interfere with a known gene-product(s), and subsequently used to probe the importance of that gene-product in a biological system. Sometimes, chemical libraries with specific functional groups are designed, as is the case with nucleotide analogues (199, 200). Successive rounds of derivatization of the lead compound are aimed to obtain a highly potent compound that is able to penetrate the cellular membrane bilayer, does not display cytotoxicity (unless desired), is not readily degraded and still retains its desired biological activity. This is done sometimes with the knowledge at hand of the three-dimensional structure of a protein (complex) that revealed an interaction- or enzymatic site, or some other ‘drugable’ pocket (200). Thus, the advantage of the reverse chemical genetic approach is that by design the target of the small molecule is known. However, it often remains a gamble whether the chemical will have the desired effect *in vivo*, or a potential use in therapeutic settings (1, 2, 201).

Forward chemical genetics: shoot first, ask later

A solution to this conundrum is offered by recent developments in high-throughout screening methodology. Perhaps the most important factor was the realization that the use of phenotypic screens could potentially overcome the hurdle of finding small molecules that display activity *in vivo*. The immediate pay-off is that in this *forward chemical genetic* approach, 'hit' compounds are selected based on an interesting biological phenotype they induce in complex systems such as cell extracts, intact cells or whole organisms. This is analogous to the identification of novel genes in *forward genetic* screens in which cells are generated with a desired phenotype by, for instance, chemical mutagenesis. As mentioned earlier, one of the two goals of chemical genetics is to identify novel molecular entities that participate in a biological process. Unfortunately, chemical target identification has met very limited success and often requires significant efforts in secondary assays and/or follow-up chemistry to overcome the notorious low affinity of the small molecules discovered (1, 2). This problem will be discussed more extensively in Chapter VI of this thesis.

Phenotypic high-throughput screens can be of quantitative nature (fluorescence, chemiluminescence, colorimetric, densitometric). The value of this approach is that by design the potency of the 'hit' compound is revealed (e.g. (37)), an important parameter to select the most useful chemicals, especially when target identification is being considered. But perhaps one of the biggest impacts in screening technology has been made by the development of automated (fluorescence) microscopy. Together with multi-parameter analysis and powerful computer algorithms, this has created the possibility of chemical profiling the spatial and temporal effect of small molecules on cellular- or subcellular behavior in a quantitative manner (202-204). Indeed, the classification of exocytosis inhibitors by the nature of their perturbation, e.g. blocking ER-versus-Golgi exit, could only be achieved because the design of the microscopy-based screen enabled the recognition of the subcellular compartment where traffic was arrested (175, 176). From the screen it became instantaneously apparent that the effect of at least some of these chemicals was reminiscent of well-characterized inhibitors of trafficking, which accelerated the elucidation of the molecular mechanisms underlying the block (175, 177). Arguably, one of the more exciting applications of microscopy-based phenotypic screening is to probe the biology of *intact* organisms, highlighted in a study of the ontogeny of the neural and cardiovascular system in zebra fish (205).

The evolution in the biological approach towards phenotypic screens came hand-in-hand with changes in the design of chemical libraries. The 'old' synthetic libraries are synthesized with the idea to stick close to molecules that worked in the past. For instance, adenosine analog libraries were designed to find novel protein kinase inhibitors (200). More recently, libraries have been engineered largely from a 'chemistry-centric' perspective, with the emphasis on creating collections that cover a large, chemical space, but that are not necessarily designed to be optimal biological agents (206). Natural product libraries are thus regaining popularity (207, 208). In addition, a relatively new and focused approach of diversity-oriented synthesis (DOS) has emerged, in which a synthetic blue print of a natural small molecule is used as a scaffold to construct a library of (natural-like) products with diverse functional groups (198, 209, 210).

Outline of this thesis

This thesis exploits the chemical genetics approach to study two different aspects of membrane biology, (a) cellular lipid transport and (b) vesicular trafficking.

Chapter II of this thesis describes the development of a cell-based quantitative high-throughput screen for SR-BI mediated selective uptake from HDL. The small molecule inhibitors identified, named BLTs because they Block Lipid Transport, were used to study the relationship between HDL binding and selective lipid uptake, and to investigate the mechanisms of selective uptake and lipid efflux.

The lipid transporters SR-BI and ABCA1 have structurally nothing in common, but they do share the ability to mediate efflux of lipids to (different) lipoprotein carriers. Small molecule inhibitors of SR-BI (BLTs) and ABCA1 (glyburide) were thus exploited to probe for the possibility that these two proteins use similar mechanisms of lipid transport (Chapter III).

Chapter IV addresses the cellular location of SR-BI mediated selective uptake with the use of BLTs and inhibitors of endocytic traffic.

In Chapter V, novel small molecule inhibitors of secretory traffic (175, 176) are used to identify elements which act as regulators of both the endocytic and the exocytic pathway.

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CHAPTER II

Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI

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Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI

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The high-density lipoprotein (HDL) receptor, scavenger receptor, class B, type I (SR-BI), mediates both the selective uptake of lipids, mainly cholesterol esters, from HDL to cells and the efflux of cholesterol from cells to lipoproteins. The mechanism underlying these lipid transfers is distinct from classic receptor-mediated endocytosis, but it remains poorly understood. To investigate SR-BI's mechanism of action and *in vivo* function, we developed a high-throughput screen to identify small molecule inhibitors of SR-BI-mediated lipid transfer in intact cells. We identified five compounds that in the low nanomolar to micromolar range block lipid transport (BLTs), both selective uptake and efflux. The effects of these compounds were highly specific to the SR-BI pathway, because they didn't interfere with receptor-mediated endocytosis or with other forms of intracellular vesicular traffic. Surprisingly, all five BLTs enhanced, rather than inhibited, HDL binding by increasing SR-BI's binding affinity for HDL (decreased dissociation rates). Thus, the BLTs provide strong evidence for a mechanistic coupling between HDL binding and lipid transport and may serve as a starting point for the development of pharmacologically useful modifiers of SR-BI activity and, thus, HDL metabolism.

The high-density lipoprotein (HDL) receptor, scavenger receptor, class B, type I (SR-BI), plays an important role in controlling the structure and metabolism of HDL (1, 2). Studies in mice have shown that alterations in SR-BI expression can profoundly influence several physiologic systems, including those involved in biliary cholesterol secretion, female fertility, red blood cell development, atherosclerosis, and the development of coronary heart disease (3–11). SR-BI controls HDL metabolism by mediating the cellular selective uptake of cholesteryl esters and other lipids from plasma HDL (1, 2). During selective uptake (12–14), HDL binds to SR-BI, and its lipids, primarily neutral lipids such as cholesteryl esters in the core of the particles, are transferred to the cells. The lipid-depleted particles subsequently are released back into the extracellular space. Although the mechanism of SR-BI-mediated selective lipid uptake and the subsequent intracellular transport of these lipids have only just begun to be explored (2, 15, 16), they clearly differ fundamentally from the pathway of receptor-mediated endocytosis via clathrin-coated pits and vesicles used by the low-density lipoprotein (LDL) receptor to deliver cholesterol esters from LDL to cells (17). SR-BI also can mediate cholesterol efflux from cells to HDL, although the physiological significance of SR-BI-mediated lipid efflux to lipoproteins is uncertain (18).

To generate reagents that can provide new insight into the mechanism of SR-BI-mediated selective lipid transfer, we have performed a high-throughput screen of a chemical library to identify potent small molecule inhibitors of SR-BI-mediated lipid transport. We report here five chemicals that block lipid transport, BLT-1–BLT-5, and describe their effects on SR-BI activity in cultured cells. All five BLTs inhibited SR-BI-mediated selective lipid uptake from HDL and efflux of cellular cholesterol to HDL. One of these, BLT-1, was particularly potent, inhibiting lipid transport in the low nanomolar concentration range. Unexpectedly, all five BLTs enhanced HDL binding to SR-BI by increasing the binding affinity. Thus, the BLTs provide strong evidence for the mechanistic coupling between HDL binding and lipid transport and should prove helpful in the analysis of the mechanism of action and function of SR-BI.

Methods

Lipoproteins and Cells. Human HDL was isolated and labeled with ^{125}I (^{125}I-HDL); 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes; DiI-HDL); or [^{3}H]cholesteryl oleyl ether ([^{3}H]CE, [^{3}H]CE-HDL) (1, 19–22). LDL receptor-deficient Chinese hamster ovary cells (ldlA-7) that express low levels of endogenous SR-BI (23), ldlA-7 cells stably transfected to express high levels of murine SR-BI (ldlA[mSR-BI]) (1), Y1-BS1 murine adrenocortical cells that express high levels of SR-BI after induction with adrenocorticotropic hormone (ACTH) (24), monkey kidney BS-C1 cells (25), and HeLa cells (26) were maintained as previously described.

High-Throughput Screen. On day 0, ldlA[mSR-BI] cells were plated at 15,000 cells per well in clear bottom, black wall, 384-well black assay plates (Costar) in 50 μl of medium A (Ham's F12 supplemented with 2 mM L-glutamine, 50 units/ml penicillin/50 μg/ml streptomycin, and 0.25 mg/ml G418) supplemented with 10% FBS (medium B). On day 1, cells were washed once with medium C [medium A with 1% (wt/vol) BSA and 25 mM Hepes, pH 7.4, but no G418] and refed with 40 μl of medium C. Compounds (16,230 from the DiverSet E, ChemBridge Corp., San Diego) dissolved in 100% DMSO were individually, robotically "pin" transferred (40 nl) by a pin-based compound transfer robot (http://iccb.med.harvard.edu) to the wells to give a nominal concentration of 10 μM (0.01% DMSO). After 1 h of incubation at 37°C, DiI-HDL (final concentration of 10 μg of protein per ml) in 20 μl of medium C was added. Two hours later, fluorescence was measured at room temperature (≈2 min per plate) with an Analyst plate reader (rhodamine B dichroic filter; excitation, 525 nm; emission, 580 nm; Molecular Devices), both before removing the incubation medium (to test for autofluorescence and quenching) and after the medium removal and four washes with 80 μl of PBS/1 mM $MgCl_2$/0.1 mM $CaCl_2$ to determine cellular uptake of DiI. All compounds were sampled in duplicate on different plates, and each screen included ldlA-7

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Abbreviations: HDL, high-density lipoprotein; SR-BI, scavenger receptor, class B, type I; LDL, low-density lipoprotein; BLT, chemical that blocks lipid transport; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; CE, cholesteryl oleyl ether; FC, free cholesterol; mSR-BI, murine SR-BI; VSV-G, vesicular stomatitis virus glycoprotein; EGFP, enhanced GFP; IC, inhibitory concentration.

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and ldlA[mSR-BI] cells in the presence or absence of a 40-fold excess of unlabeled HDL, but with no added compounds, as controls.

Assays. For the assays, all media and buffers contained 0.5% DMSO and 0.5% BSA to maintain compound solubility. Cells were preincubated with BLTs for 1 h (or 2.5 h for transferrin, epidermal growth factor, and cholera toxin uptake experiments), and all of the experiments were performed at 37°C unless otherwise noted. Detailed characterization of the BLTs and their effects was performed with compounds whose identities and purities were confirmed by LC-MS.

Lipid uptake from HDL, cholesterol efflux to HDL, and HDL binding assays. Assays for the uptake of lipids from DiI-HDL and [^{3}H]CE-HDL, efflux of [^{3}H]cholesterol from labeled cells, and ^{125}I-HDL binding were performed as described (1, 18, 20). In some experiments, values were normalized so that 100% of control represents activity in the absence of compounds and 0% represents activity determined in the presence of a 40-fold excess of unlabeled HDL or, for Y1-BS1 cells, in the presence of a 1:500 dilution of the KKB-1 blocking antibody (generous gift from Karen Kozarsky, Glaxo/SmithKline Beecham, King of Prussia, PA) (20). The amounts of cell-associated [^{3}H]cholesteryl oleyl ether are expressed as the equivalent amount of [^{3}H]CE-HDL protein (ng) to permit direct comparison of the relative amounts of ^{125}I-HDL binding and [^{3}H]CE uptake.

The rates of HDL dissociation from cells were determined by incubation of the cells with ^{125}I-HDL (10 μg of protein per ml, 2 h, 37°C) with and without BLTs. Then either the medium was replaced with the same medium in which a 40-fold excess of unlabeled HDL was substituted for the ^{125}I-HDL, or a 40-fold excess of unlabeled HDL was added to the labeled incubation medium. The amounts of cell-associated ^{125}I-HDL then were determined as a function of time. The two methods gave similar results.

Fluorescence microscopic analysis of intracellular trafficking and cytoskeletal organization. Receptor-mediated endocytosis of Alexa-594-labeled transferrin or FITC-labeled epidermal growth factor (Molecular Probes) by HeLa cells (27) and uptake of Alexa-594-labeled holo-cholera toxin (kind gift of Wayne Lencer, Children's Hospital Boston and Harvard Medical School) by BSC-1 cells were detected by fluorescence microscopy. The intracellular transport of the temperature-sensitive glycoprotein of vesicular stomatitis virus (VSV-G^{ts045}), fused at its carboxyl terminus to enhanced GFP (VSV-G^{ts045}-EGFP) from the endoplasmic reticulum to the plasma membrane, after a shift from 40 to 32°C for 2 h, was determined by fluorescence microscopy (Y. Feng, S. Yu, T. Lasell, A. Jadhav, E. Macia, P. Chardin, P. Melancon, M. Roth, T. Mitchison, and T.K., unpublished data). The effects of the compounds on the distribution of actin using rhodamine-labeled phalloidin and tubulin using the FITC-labeled DM1α monoclonal antibody (Sigma) in ldlA[mSR-BI] cells were determined as described (25) by fluorescence microscopy using an air $\times$63 objective (Nikon).

Flow cytometric analysis of SR-BI cell surface expression. Cells were incubated for 3 h (medium C) with or without BLTs at their IC_{95CE} concentrations and harvested with PBS containing 2 mM EDTA and compounds, and the levels of SR-BI surface expression in unfixed cells were determined at 4°C by flow cytometry with the KKB-1 antibody (19).

Results

High-Throughput Screening for Inhibitors of SR-BI-Mediated Selective Lipid Uptake. Cellular uptake and accumulation of the fluorescent lipophilic dye DiI from DiI-labeled HDL (DiI-HDL) is a reliable surrogate for SR-BI-dependent selective uptake of the cholesteryl esters in HDL (1). To identify small molecule inhibitors of SR-BI-mediated selective lipid uptake, we screened 16,320 compounds representing the DiverSet E of the ChemBridge library collection for their abilities to block the cellular uptake of DiI from DiI-HDL. The compounds were tested at a nominal concentration of 10 μM in a 384-well plate assay using ldlA[mSR-BI] cells that express a high level of mSR-BI (1). Fig. 1 shows results from a representative assay plate along with controls (no compounds, addition of excess unlabeled HDL or use of untransfected ldlA-7 cells). Compounds that quenched (Q) or enhanced the intrinsic fluorescence of DiI-HDL were not examined further. Approximately 200 compounds that reproducibly blocked DiI uptake in a first round of screening were retested. Five of the most effective with IC_{50DiI}s in the micromolar range or lower (Fig. 2*A*) were designated BLT-1–BLT-5 and further characterized. Strikingly, the most potent of these, BLT-1 and BLT-2, inhibited in the nanomolar range and are structurally related (Table 1). Inhibition of DiI uptake did not require *de novo* protein synthesis, because pretreatment of cells for 30 min with 100 μg/ml cycloheximide did not diminish their inhibitory effects (data not shown). Finally, there were essentially no effects of the BLTs on the low background level of uptake of DiI or [^{3}H]CE by untransfected ldlA-7 cells expressing minimal amounts of SR-BI (data not shown).

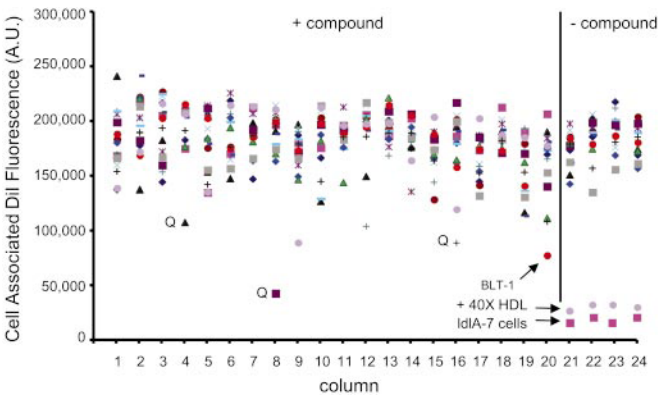


Fig. 1. High-throughput screen for inhibitors of SR-BI-mediated DiI uptake from DiI-HDL. Example of a fluorescent readout obtained from a single 384-well plate during the first round of the high-throughput screen. SR-BI-expressing ldlA[mSR-BI] cells were plated into 384-well plates, and the effect of compounds (≈10 μM) on the uptake of DiI from DiI-HDL (10 μg of protein per ml) was determined by using a high-speed fluorescence plate reader. Columns 1–20 show results (fluorescence in arbitrary units) from 16 independent wells per column (different colored symbols) from a single plate, representing a total of 320 compounds. Controls without compounds are wells containing ldlA[mSR-BI] cells in the absence or presence of a 40-fold excess of unlabeled HDL, or containing untransfected ldlA-7 cells (very low SR-BI expression). Wells containing an inhibitory compound named BLT-1 and wells with compounds that quenched DiI-HDL fluorescence (Q) are indicated.

The IC_{50CE}s for inhibition of uptake of the more physiologic lipid [^{3}H]cholesteryl oleyl ether ([^{3}H]CE) from [^{3}H]CE-HDL by ldlA[mSR-BI] cells were similar to those for DiI uptake (Fig. 2*B* and Table 1). The inhibition of [^{3}H]CE uptake was reversible (1 h of incubation with compounds followed by a 3- to 6-h washout period and a subsequent 2-h incubation with [^{3}H]CE-HDL, data not shown). Moreover, the compounds also blocked the uptake of [^{3}H]CE by Y1-BS1 adrenocortical cells that express high levels of SR-BI (24) (Table 1), indicating that the inhibitory effects by the compounds are not cell-type specific. Experiments in which the cells or the labeled HDL were preincubated with the compounds indicated that the cells, rather than the HDL, were the target of the compounds (data not shown).

Inhibition of Selective Lipid Uptake by BLTs Is Specific. We examined the specificity of BLT inhibition by testing the effects of the BLTs on several other cellular properties at concentrations that inhibit

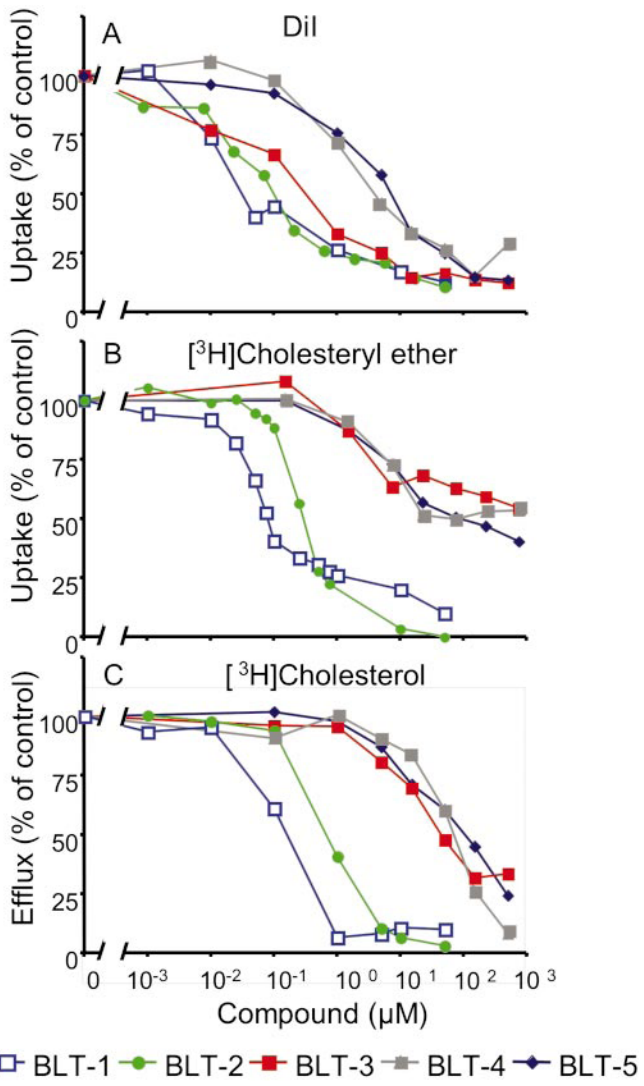


Fig. 2. Concentration dependence of the inhibition by BLTs of SR-BI-mediated lipid transfer between HDL and cells. ldlA[mSR-BI] cells were incubated with the indicated concentrations of BLTs, and their effects on DiI uptake from DiI-HDL (*A*), [^{3}H]CE uptake from [^{3}H]CE-HDL (*B*), and the efflux of [^{3}H]cholesterol from cells to HDL (*C*) were determined. The 100% of control values were 50.6 ng of HDL protein equivalents per well (384-well plates) (*A*) and 3,908 ng of HDL protein equivalents per mg of cellular protein (*B*). In *C*, the data were normalized such that the maximum amount of [^{3}H]cholesterol transferred from cells to HDL in the absence of compounds (55.7% of total) was set to 100%. The 0% value corresponds to the efflux of [^{3}H]cholesterol transferred from ldlA[mSR-BI] cells to HDL without BLTs and in the presence of saturating inhibitory amounts of the specific anti-SR-BI blocking antibody KKB-1 (15% of total) (20). The efflux of [^{3}H]cholesterol from ldlA-7 cells measured in the absence or presence of KKB-1 was 15% and 10% of total cellular [^{3}H]cholesterol, respectively.

[^{3}H]CE uptake by 95% (IC_{95CE}) (Fig. 3). None of the BLTs disrupted the integrity of the actin and tubulin networks. They also did not inhibit the uptake or alter the intracellular distribution of the fluorescently labeled endocytic receptor ligands transferrin and epidermal growth factor. The BLTs also failed to inhibit the uptake of fluorescently labeled cholera toxin from the cell surface to perinuclear regions through a pathway believed to depend in part on cholesterol- and sphingolipid-rich lipid rafts (28). Moreover, BLTs did not interfere with the secretory pathway, as assessed by analysis of the transport of VSV-G^{ts045}-EGFP. Thus, BLTs do not induce general defects in clathrin-dependent and -independent intracellular membrane trafficking or in the organization of the cytoskeleton and are, by these criteria, specific inhibitors of SR-BI-dependent lipid uptake.

BLTs Inhibit SR-BI-Mediated Cholesterol Efflux from Cells to HDL. In addition to mediating selective lipid uptake from HDL, SR-BI can facilitate the efflux of unesterified, or free, cholesterol (FC) from cells to HDL particles (18). To determine whether the BLTs could inhibit this SR-BI-mediated lipid transport activity, we labeled cells with [^{3}H]cholesterol and measured its efflux to unlabeled HDL in the presence or absence of the BLTs (Fig. 2*C* and Table 1). All BLTs inhibited SR-BI-mediated cholesterol efflux with relative potencies (IC_{50FC}s) similar to those for [^{3}H]CE uptake; however, in the cases of BLT-3, BLT-4, and BLT-5, the IC_{50FC}s for efflux were higher than those for uptake, suggesting that the BLTs may have uncovered possible differences in the mechanisms of uptake and efflux. In ldlA[mSR-BI] cells, the BLTs had little effect on the SR-BI-independent efflux (not inhibited by the specific anti-SR-BI blocking antibody KKB-1; ref. 20) (data not shown). Similarly, in untransfected ldlA-7 cells expressing relatively low levels of endogenous SR-BI, total and SR-BI-dependent (e.g., KKB-1-inhibitable) cholesterol efflux was substantially lower than in ldlA[mSR-BI] cells. The BLTs were able to inhibit the low SR-BI-dependent cholesterol efflux in ldlA-7 cells, but they had no inhibitory effect on the similarly low SR-BI-independent efflux (data not shown).

BLTs Do Not Change the Surface Expression of SR-BI. To determine whether BLTs inhibited SR-BI function by reducing its cell surface expression, we measured surface expression by using the KKB-1 anti-mSR-BI antibody (20) and flow cytometry. Fig. 4 shows that, after 3 h of incubation at their IC_{95CE}s (corresponding to 1 μM for BLT-1 and BLT-2 and 50 μM for BLT-3–BLT-5), the BLTs did not alter the expression of mSR-BI on the surfaces of ldlA[mSR-BI] cells.

BLTs Enhance Binding of HDL to SR-BI. We initially expected that the BLTs would function by inhibiting HDL binding to SR-BI. However, when cells were incubated with a subsaturating concentration of either [^{3}H]CE-HDL or ^{125}I-labeled HDL (^{125}I-HDL) (10 μg of protein per ml) and increasing amounts of compound (Fig. 5), the decreases in [^{3}H]CE uptake (solid lines, no symbols, data from Fig. 2*B*) and [^{3}H]cholesterol efflux (dashed lines, data from Fig. 2*C*) were accompanied by corresponding increases in ^{125}I-HDL binding (Fig. 5, solid lines). The concentration dependence of ^{125}I-HDL binding was determined in the presence or absence of BLTs at their IC_{95CE} concentrations (Fig. 6 and Table 1). The BLTs did not substantially alter the number of binding sites (B_{max}), but rather induced small, yet significant, increases in the affinity of SR-BI for HDL (lower apparent K_d). Furthermore, the BLTs reduced the rates of dissociation of ^{125}I-HDL from SR-BI (Table 1), indicating that the tighter binding induced by the BLTs was due, at least in part, to a decrease in the dissociation rate.

Discussion

In this study, we report the discovery and characterization of BLT-1–BLT-5, small molecules that inhibit the transfer of lipids between HDL and cells mediated by the HDL receptor SR-BI. BLTs inhibited both cellular selective lipid uptake of HDL cholesteryl ether and efflux of cellular cholesterol to HDL. The inhibitory effects of the BLTs were specific, because they required SR-BI and they did not interfere with several clathrin-dependent and -independent endocytic pathways, the secretory pathway, or the actin or tubulin cytoskeletal networks. Strikingly, inhibition of lipid transfer by BLTs was accompanied by enhanced HDL binding affinity (reduced dissociation rates).

Table 1. Effects of BLTs on SR-BI activity

	n	BLT-1, mean ± SD	BLT-2, mean ± SD	BLT-3, mean ± SD	BLT-4, mean ± SD	BLT-5, mean ± SD	No BLT, mean ± SD
IC_{50}, μM							
DiI-HDL uptake	3	0.06 ± 0.04	0.35 ± 0.18	0.51 ± 0.15	2.0 ± 1.0	7.1 ± 3.7	— —
[^{3}H]CE-HDL uptake							
ldlA[mSR-BI] cells	6	0.11 ± 0.08	0.24 ± 0.1	2.3 ± 1.5	3.9* ± 0.76	13.8* ± 8.5	— —
Y1-BS1 cells	2	0.38†	0.41†	1.7†	4.4†	8.0†	— —
[^{3}H]cholesterol efflux	3	0.15 ± 0.09	0.47 ± 0.23	17.2 ± 4.0	54.9 ± 35.2	75.3 ± 40.1	— —
^{125}I-HDL binding	3	0.088 ± 0.05	0.25 ± 0.13	46.5 ± 49.3	24.9 ± 14.8	18.0 ± 3.7	— —
Binding parameters							
Apparent K_d, μg·ml^{-1}	3	4.7 ± 0.05	6.0 ± 6.0	8.0 ± 4.0	8.9 ± 2.3	12.0 ± 1.6	16.6 ± 1.5
K_{off}, min^{-1}	2	0.06†	0.062†	0.08†	0.082†	0.079†	0.11†
B_{max}, %		95.8 ± 10.1	93.0 ± 20.5	85.8 ± 15.8	79.9 ± 15.9	92.1 ± 36.8	100.0 ± 18.4

All experiments were with ldlA[mSR-BI] cells except where noted.
*n = 5.
†SD is not applicable.

BLTs and other inhibitors or activators of SR-BI should be useful for the analysis of the molecular and cellular mechanisms of SR-BI activity and the study of the physiologic functions of SR-BI by its pharmacologic manipulation *in vivo*. Our studies with the BLTs support a two-step mechanism of SR-BI activity, productive binding followed by lipid transfer (19, 29), and suggest that these two steps may be mechanistically linked because they result in coordinated decreases in lipid transfer and increases in binding affinity. They also suggest that there may be differences in the mechanisms of SR-BI-mediated lipid uptake and cholesterol efflux (20, 30), because the IC_{50} values for uptake and efflux for each of three BLTs (BLT-3–BLT-5) differed.

The mechanistic coupling of HDL binding and lipid transport observed here is consistent with the productive binding model for SR-BI interaction with HDL (29). A number of studies have explored the influence of the protein composition of HDL particles on binding and lipid transfer (26, 29, 31–33). They showed that variations in the structure/composition of HDL particles could affect the efficiency of selective uptake or efflux relative to lipoprotein binding and thus support the concept of productive binding. In addition, CD36, a close homolog of

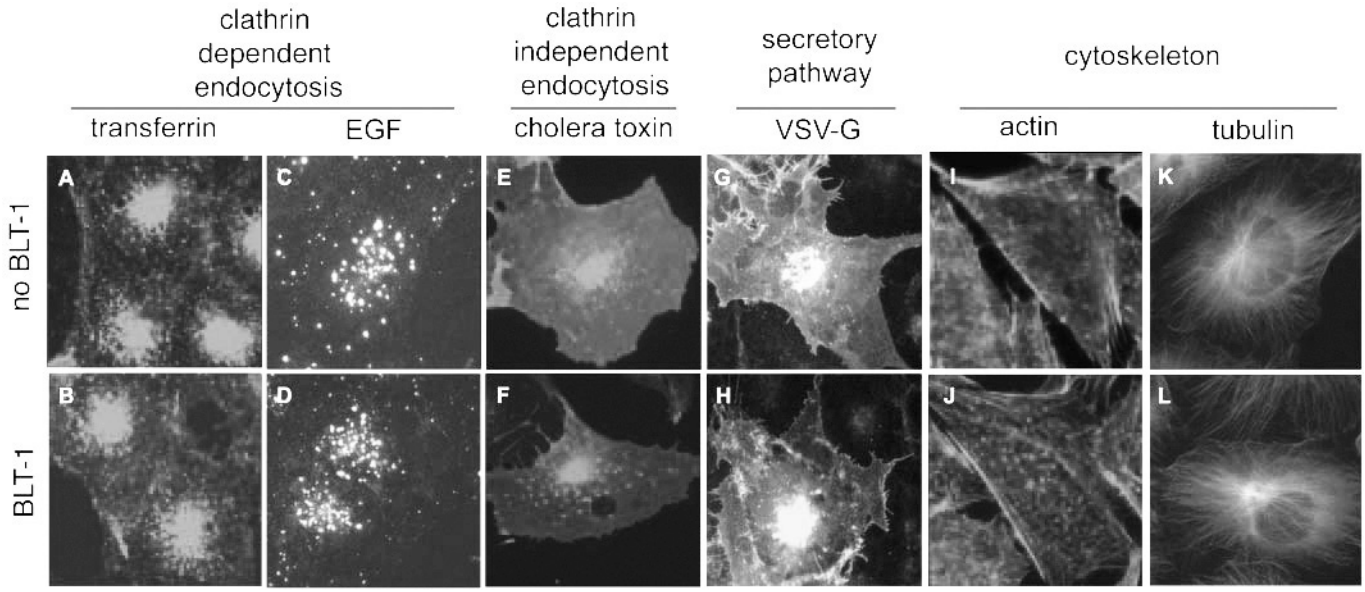
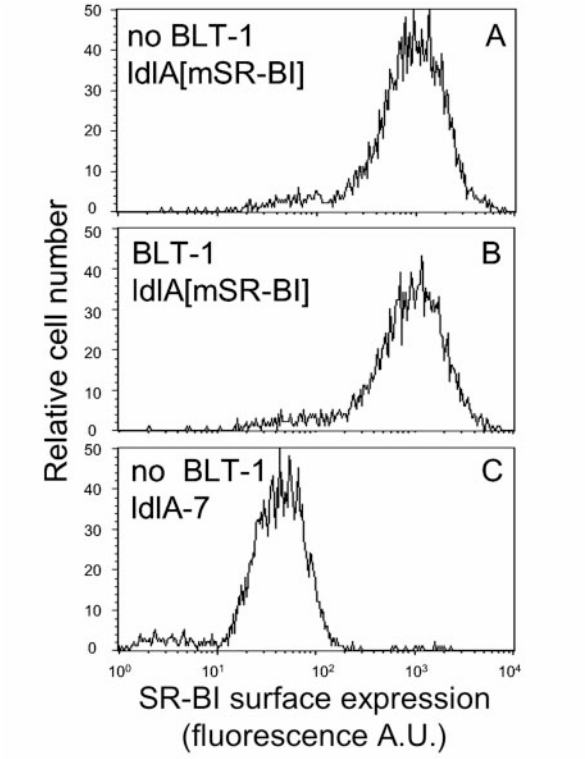


Fig. 3. Effects of BLT-1 on intracellular membrane trafficking and cytoskeletal organization. Cells were incubated for 3 h in the absence (*Upper*) or presence (*Lower*) of 50 μM BLT-1, and epifluorescence light microscopy was used to monitor the following cellular activities: clathrin-dependent endocytosis of fluorescently labeled transferrin (*A* and *B*; HeLa cells) and epidermal growth factor (*C* and *D*; HeLa cells); clathrin-independent endocytosis of fluorescently labeled cholera toxin (*E* and *F*; BSC-1 cells); and transport of the temperature-sensitive fluorescent membrane protein VSV-G^{ts045}-EGFP from the endoplasmic reticulum to the cell surface (*G* and *H*; BSC-1 cells). In addition, the intracellular distributions of the actin cytoskeleton (*I* and *J*; ldlA-[mSRBI] cells visualized with rhodamine-labeled phalloidin) and the tubulin network (*K* and *L*; BSC-1 cells visualized with fluorescently labeled antibodies specific to α-tubulin) were determined. BLT-1 and the other BLTs (data not shown) had no effects on any of these cellular properties or activities.



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compound	n	% SR-BI surface expression ± SD
no BLT	6	100.0
BLT-1	6	94.4 ± 4.5
BLT-2	4	99.6 ± 10.1
BLT-3	3	91.7 ± 10.7
BLT-4	3	97.2 ± 5.1
BLT-5	5	102.5 ± 2.7

Fig. 4. Cell surface expression of SR-BI. IdlA[mSR-BI] and IdlA-7 cells were treated for 3 h with or without BLTs at their corresponding IC_{95CE} concentrations (1 μM for BLT-1 and BLT-2 and 50 μM for BLT-3–BLT-5) followed by determination of surface expression levels of SR-BI by flow cytometry. *A–C* show histograms of the surface expression for IdlA[mSR-BI] cells without BLTs, IdlA[mSR-BI] cells with 1 μM BLT-1, and IdlA-7 cells without BLTs, respectively. *D* summarizes the results in IdlA[mSR-BI] cells for all five BLTs, with the value determined without compounds set to 100%. n, number of independent determinations.

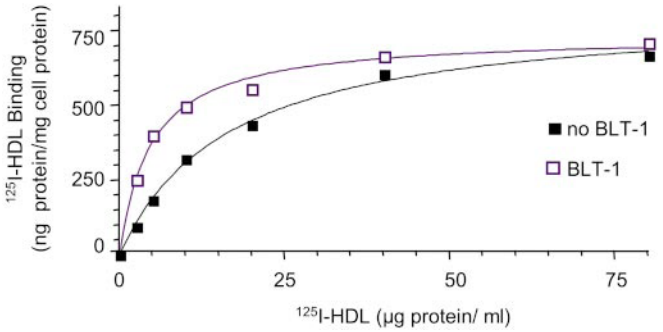

Fig. 6. Effects of BLT-1 on the concentration dependence of ^{125}I-HDL binding to IdlA[mSR-BI] cells. The binding of ^{125}I-HDL to IdlA[mSR-BI] cells was determined in duplicate at the indicated concentrations of HDL in the presence (blue) or absence (black) of 1 μM BLT-1 (IC_{95CE}). Each value was corrected for binding of ^{125}I-HDL in the presence of 40-fold excess of unlabeled HDL to IdlA [mSR-BI] cells in the presence of BLT-1.

SR-BI, mediates high-affinity HDL binding but not efficient lipid transfer (19, 34). Thus, HDL binding *per se* to a member of this superfamily of receptors is not sufficient for efficient lipid transport; rather, the binding must be productive. That is, SR-BI and HDL must bind in a precisely aligned fashion, have the capacity to undergo appropriate conformational changes on binding, or both.

BLTs might block productive binding by inducing (or preventing) interactions between HDL and its receptor, or interfering with critical conformational changes such that lipid transfer could not occur. A direct consequence of these altered interactions could be the tighter HDL binding observed when the cells were treated with BLTs. Alternatively, the BLTs might not interfere with productive binding and lipid transfer, but rather might simply inhibit dissociation of HDL from SR-BI, thereby preventing the multiple rounds of HDL binding, lipid transfer, dissociation of the smaller lipid-depleted particle, and binding of a new HDL particle that are required for efficient selective uptake. If this were the case, in the presence of BLTs the initially bound HDL particles would competitively inhibit the binding of other particles. Another possibility is that direct BLT inhibition of the lipid transfer step might prevent the size reduction of HDL that accompanies selective uptake. Previous studies have shown that larger, lipid-rich HDLs bind more tightly to SR-BI than smaller, relatively lipid-poor HDLs (33, 35). Thus, BLT inhibition of the lipid transfer step would lead to larger HDL particles bound more tightly to SR-BI at steady state, resulting in a slower dissociation rate and apparently higher binding affinity. Finally, it seems unlikely that the BLTs would inhibit lipid transfer step(s) occurring distally to the receptor itself [e.g., between the

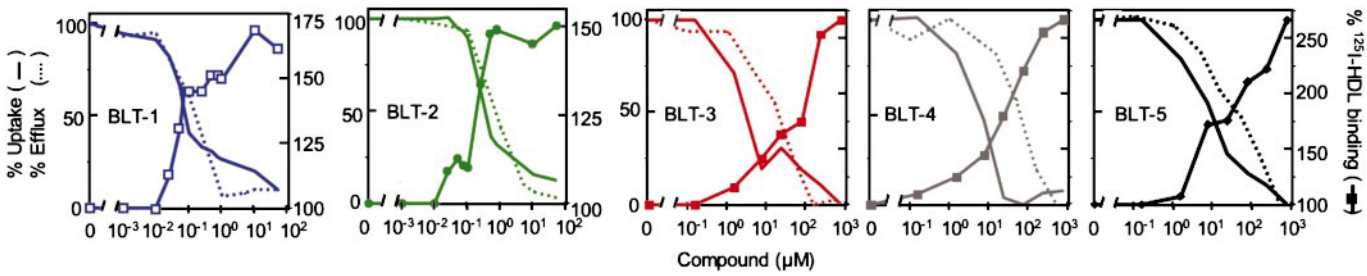

Fig. 5. Effects of BLTs on SR-BI-mediated cholesterol ether uptake from HDL, cellular cholesterol efflux to HDL, and HDL binding. The effects of the indicated concentrations of BLTs on SR-BI-mediated uptake of [^{3}H]CE from [^{3}H]CE-HDL (solid lines, no symbols), efflux of [^{3}H]cholesterol from cells to HDL (dashed lines), or binding of ^{125}I-HDL to cells (solid lines, symbols) were determined by using IdlA[mSR-BI] cells. To simplify comparisons, we define the lowest observed [^{3}H]CE uptake and [^{3}H]cholesterol efflux values (from Fig. 2) as 0% and the values in the absence of BLTs as 100%. The 100% control value for the ^{125}I-HDL binding in the absence of BLTs was 403 ng of HDL protein per mg of cell protein.

plasma membrane and internal cellular compartments significantly downstream from the association between HDL and SR-BI (16)], given the strong inverse correlation between the effects of BLTs on binding and lipid transport. Future identification of the molecular target(s) of the BLTs, whether SR-BI or other proteins or lipids, should provide insights into the mechanisms underlying the activities of both the BLTs and SR-BI.

Experiments with SR-BI-deficient or SR-BI-overexpressing mice have provided important insights into SR-BI function and potential roles of SR-BI and HDL metabolism in pathophysiology, including female infertility, aberrant red blood cell development, and cardiovascular disease (6, 7, 10, 15). BLTs and other small molecules isolated by using the approach described here may prove helpful in further studying the physiology of these systems. In this regard it is noteworthy that BLT-1 has been shown to interfere with the early stages of brain development in zebrafish (36). It may be worthwhile to explore the potential role of disrupted lipid metabolism in this system. Given the widespread consequences of genetically manipulating SR-BI expression in mice, pharmacologic manipulation may have potential therapeutic value.

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CHAPTER III

Cross-inhibition of SR-BI- and ABCA1-mediated cholesterol transport by the small molecules BLT-4 and glyburide

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Cross-inhibition of SR-BI- and ABCA1-mediated cholesterol transport by the small molecules BLT-4 and glyburide

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Abstract Scavenger receptor class B type I (SR-BI) and ABCA1 are structurally dissimilar cell surface proteins that play key roles in HDL metabolism. SR-BI is a receptor that binds HDL with high affinity and mediates both the selective lipid uptake of cholesteryl esters from lipid-rich HDL to cells and the efflux of unesterified cholesterol from cells to HDL. ABCA1 mediates the efflux of unesterified cholesterol and phospholipids from cells to lipid-poor apolipoprotein A-I (apoA-I). The activities of ABCA1 and other ATP binding cassette superfamily members are inhibited by the drug glyburide, and SR-BI-mediated lipid transport is blocked by small molecule inhibitors called BLTs . Here, we show that one BLT, [1-(2-methoxy-phenyl)-3-naphthalen-2-yl-urea] (BLT-4), blocked ABCA1-mediated cholesterol efflux to lipid-poor apoA-I at a potency similar to that for its inhibition of SR-BI ($IC_{50} \sim$ 55–60 μM). Reciprocally, glyburide blocked SR-BI-mediated selective lipid uptake and efflux at a potency similar to that for its inhibition of ABCA1 ($IC_{50} \sim$275–300 μM). As is the case with BLTs, glyburide increased the apparent affinity of HDL binding to SR-BI. The reciprocal inhibition of SR-BI and ABCA1 by BLT-4 and glyburide raises the possibility that these proteins may share similar or common steps in their mechanisms of lipid transport.—Nieland, T. J. F., A. Chroni, M. L. Fitzgerald, Z. Maliga, V. I. Zannis, T. Kirchhausen, and M. Krieger. **Cross-inhibition of SR-BI- and ABCA1-mediated cholesterol transport by the small molecules BLT-4 and glyburide *J. Lipid Res.* 2004.** 45: **1256–1265.**

Supplementary key words scavenger receptor class B type I • ATP binding cassette transporter A1 • blockers of lipid transport

Cholesterol and cholesteryl ester movement into and out of cells mediated by lipoproteins and cell surface proteins such as scavenger receptor class B type I (SR-BI) and ABCA1 plays an important role in cellular and whole body lipid metabolism and can profoundly influence the risk of atherosclerosis and coronary heart disease (1–8). The pathologic consequences of naturally arising or experimentally induced disruptions in the SR-BI and ABCA1 genes clearly demonstrate that these lipid transport proteins serve physiologically important, yet dramatically distinct, purposes in HDL metabolism (1, 2).

SR-BI, a member of the CD36 superfamily of proteins, is a 509 residue glycoprotein (apparent mass of 82 kDa) containing two predicted transmembrane helices adjacent to very short N- and C-terminal cytoplasmic domains, with the bulk of the protein consisting of a large extracellular loop (3, 4). Alterations in SR-BI expression can influence profoundly several physiologic processes, including biliary cholesterol secretion, female fertility, red blood cell maturation, and the development of atherosclerosis and coronary heart disease (5–7, 9–14). SR-BI tightly binds large, spherical, cholesteryl ester-rich HDL particles, primarily via its major apolipoprotein, apolipoprotein A-I (apoA-I), but lipid-free apoA-I is a poor ligand (15). SR-BI mediates the selective uptake of HDL cholesteryl esters (16–19) and other lipids (3, 20, 21) into cells, after which the lipid-depleted particles dissociate from the cells. The mechanism of selective lipid uptake differs markedly from that of the classic coated pit-mediated endocytic uptake of LDL receptors (22). The binding of native spherical HDL par-

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Abbreviations: ABC, ATP binding cassette; apoA-I, apolipoprotein A-I; BLT, small molecules that block lipid transport; COE, cholesteryl oleyl ether; DSP, dithiobis (succinimidyl propionate); K_d, dissociation constant; SR-BI, scavenger receptor class B type I; SUR, sulfonylurea receptor.

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ticles or reconstituted apoA-I/phospholipid/cholesterol disks to SR-BI also results in the efflux of unesterified cholesterol from cells to the particles (23–25), but the physiological significance of this activity has not yet been determined.

ABCA1, a member of the ATP binding cassette (ABC) superfamily of proteins, has a structure strikingly different from that of SR-BI. ABCA1 is a much larger protein (~200 kDa) that contains 12 membrane-spanning helices, two cytoplasmic ATP binding domains, and two large extracellular loops (2, 26, 27). It mediates the transfer of unesterified cholesterol and phospholipids from cells to lipid-poor apoA-I (28–31), which can subsequently mature into spherical, cholesteryl ester-rich HDL particles (32–35). These mature HDLs are poor acceptors for ABCA1-mediated cholesterol efflux (36). Mutations in ABCA1 are the underlying cause of Tangier disease, which is characterized by the virtual absence of spherical, cholesteryl ester-rich HDL particles in the plasma and an accumulation of cholesterol in peripheral macrophages (28, 37–39). Mice with targeted homozygous inactivating mutations in the ABCA1 gene exhibit phenotypes similar to those of human Tangier patients (40–42). In contrast, SR-BI-deficient mice (homozygous null mutations) have increased levels of abnormally large lipid-rich HDL particles in their plasma (43). The differences in the structures of SR-BI and ABCA1 suggest that they use different mechanisms to transfer lipids between cells and HDL. This is supported by experiments showing that the structural or conformational determinants on apoA-I that are crucial for its interaction with ABCA1 (e.g., apoA-I's C terminus) appear to differ from those necessary for productive interactions with SR-BI (15, 24, 44). However, their common abilities to mediate cholesterol efflux from cells led us to probe their activities using small molecules that had previously been shown to inhibit their lipid transport activities. These included small molecules that block lipid transport (BLTs) mediated by SR-BI (45) and the drug glyburide (29, 31, 46–51), which blocks ABCA1-mediated cholesterol efflux.

Glyburide (also called glybenclamide; **Fig. 1A**) is a sulfonylurea that has received wide attention in clinical settings as a potent drug for the treatment of non-insulin-dependent diabetes mellitus (52). Glyburide binds tightly to and inhibits sulfonylurea receptors 1 and 2 (SUR1 or SUR2), members of the ABC superfamily of proteins that form the SUR subunit of the ATP-sensitive potassium channel. SUR1 and SUR2 are responsible for the binding and hydrolysis of ATP and effectively control the closing of the channel and the regulation of insulin release. The dissociation constant (K_d) for glyburide binding to SUR1, which apparently involves association with its transmembrane domains, is in the low nanomolar range [reviewed in ref. (53)]. Glyburide also inhibits the activities of other ABC proteins (54–56), including ABCA1. Inhibition of ABCA1 by glyburide has been reported to occur in the concentration range of 100–1,000 μM (29, 31, 46–51).

By using a high-throughput chemical library screen, we recently identified five small molecules (BLT-1 to BLT-5)

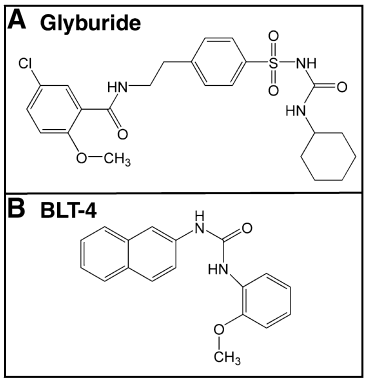

Fig. 1. Chemical structures of glyburide (A) and 1-(2-methoxyphenyl)-3-naphthalen-2-yl-urea (BLT-4) (B).

that in the low nanomolar to micromolar range block lipid transport (selective uptake from and efflux to HDL) mediated by SR-BI (45). Unexpectedly, they enhance rather than decrease the apparent affinity of HDL binding to SR-BI.

Here, we report that 1-(2-methoxy-phenyl)-3-naphthalen-2-yl-urea (BLT-4) (Fig. 1B) blocks ABCA1-mediated cholesterol efflux to lipid-free apoA-I. The other BLTs did not inhibit ABCA1 activity. Conversely, glyburide prevented SR-BI-mediated selective lipid uptake from and cholesterol efflux to HDL. Similar to the BLTs, glyburide enhanced the affinity of HDL binding to SR-BI. The concentrations at which glyburide inhibited SR-BI- and ABCA1-mediated lipid efflux were similar (IC_{50} ~ 275–300 μM), as were those for BLT-4, but BLT-4 was the more potent inhibitor (IC_{50} ~ 55–60 μM). The reciprocal or cross-inhibition of these two lipid transport proteins by BLT-4 and glyburide raises the possibility that there may be similarities in at least one step of the mechanisms of SR-BI- and ABCA1-mediated lipid transport

EXPERIMENTAL PROCEDURES

Stock solutions of BLTs (Chembridge Corp.) and glyburide (Sigma) were prepared in 100% DMSO and diluted into the assay medium immediately before use. Dithiobis (succinimidyl propionate) (DSP; from Pierce Biotechnology, Inc.), which contains thiol-sensitive bonds, was dissolved in dimethyl sulfoxide and then diluted to 250 μM with PBS immediately before use.

Lipoproteins and cells

Human HDL (density of ~1.09–1.16 g/ml) and recombinant lipid-free human apoA-I were isolated and labeled with either ^{125}I (^{125}I-HDL or ^{125}I-apoA-I) or [^{3}H]cholesteryl oleyl ether ([^{3}H]COE, [^{3}H]COE-HDL) (3, 19, 25, 44, 57, 58). LDL receptor-deficient Chinese hamster ovary cells that express low levels of endogenous SR-BI (ldlA-7) (59), ldlA-7 cells stably transfected to express high levels of murine SR-BI (ldlA[mSR-BI]) (19), and HEK293-EBNA-T human embryonic kidney (HEK) cells (27) were maintained in culture as previously described. All assay media contained 0.5% or 0.29% (v/v) DMSO and 0.5% (w/v) BSA (fatty acid-free form in efflux assays), and all experiments were conducted at 37°C.

Lipid transport and binding assays

All data presented are representative of results from two or more independent experiments.

SR-BI. Assays for the uptake of [^{3}H]COE from [^{3}H]COE-HDL, efflux of [^{3}H]cholesterol from labeled cells, and ^{125}I-HDL binding were performed using ldlA[mSR-BI] cells and control untransfected ldlA-7 cells as previously described (19, 23, 25). We also determined the SR-BI-mediated efflux of [^{3}H]cholesterol from HEK293-EBNA-T cells transiently transfected with an SR-BI expression plasmid (19), as described for ABCA1 below. On the day of the assay, cells were preincubated for 1 h at 37°C in assay medium (Ham's F12, 0.5% BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4) containing compounds at the indicated concentrations. Subsequently, the cells were incubated for an additional 2 h with the same concentration of small molecules and with the indicated concentrations of ^{125}I-HDL (binding), [^{3}H]COE-HDL (uptake), or unlabeled HDL (efflux). ^{125}I-HDL saturation binding assays were performed as previously described, in which a 40-fold excess of unlabeled HDL was included in some of the incubations to permit the correction for nonspecific binding (19, 45). Analysis of saturation binding curves was performed using GraphPad Prism3 software from GraphPad Software, Inc. (San Diego, CA).

For some experiments, the values presented were normalized so that 100% of control represents activity in the absence of compounds. In Figs. 2 and 3B, 0% activity was defined as the activity determined in the presence of a 1:800 dilution of the anti-SR-BI blocking antibody KKB-1 (25), a generous gift from Karen Kozarsky. In other experiments, the 0% values were defined as the amount of activity either in ldlA-7 control cells in the presence of drugs (Fig. 4C) or in ldlA[mSR-BI] cells in the presence of a 40-fold excess of unlabeled HDL (Fig. 5). The amount of cell-associated [^{3}H]COE is expressed as the equivalent amount of [^{3}H]COE-HDL protein (nanograms) to permit direct comparison of the relative amounts of ^{125}I-HDL binding and [^{3}H]COE uptake (60).

ABCA1. ABCA1-dependent efflux of [^{3}H]cholesterol was measured using HEK293-EBNA-T cells transiently transfected with either an ABCA1 pcDNA1 expression vector or the empty pcDNA1 vector control, as previously described (44, 61). On day 0, HEK293-EBNA-T cells were plated at 200,000 cells/well on 24-well poly-D-lysine-coated plates (Becton Dickinson) in medium A [DMEM containing high glucose and 10% (v/v) fetal calf serum without antibiotics]. On day 1, cells were transfected with either the ABCA1 vector or an "empty" vector control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggestions. The media were removed 16 h later, and cells were incubated for 24 h in medium A supplemented with 0.5 μCi/ml 1,2[^{3}H]cholesterol (Perkin-Elmer). The cells were then washed twice with DMEM (high glucose) and incubated for 1 h in assay medium [DMEM (high glucose) supplemented with 0.2% fatty acid-free BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4]. The cells were then pretreated with the indicated concentrations of small molecules in the assay medium for 1 h at 37°C and then incubated with the same concentrations of compounds in the presence or absence of 10 μg/ml lipid-free recombinant human apoA-I for an additional 4 h to permit the efflux of cellular cholesterol. The supernatant was harvested and clarified by centrifugation (5 min, 6,000 g), the cells were lysed in 0.1 M NaOH, and radioactivity in both fractions was measured by liquid scintillation counting.

Cholesterol efflux was defined as the amount of radioactivity in the extracellular media at the end of the 4 h incubation divided by the total amount of cellular radioactivity in the media plus cells (the percentage efflux). The apoA-I-dependent efflux is defined as the difference between the efflux values determined for incubations in medium that did or did not contain lipid-free apoA-I. The ABCA1- and apoA-I-dependent efflux is defined as the difference between the apoA-I-dependent efflux values determined using cells that express the ABCA1 transgene (HEK[ABCA1]) and the empty vector controls (HEK[control]). Efflux values are presented as the percentage of total cellular 1,2[^{3}H]cholesterol present in the cells at the beginning of the 4 h incubation.

The IC_{50} values are those concentrations of the compounds that caused a half-maximal inhibition of lipid transport mediated by either ABCA1 or SR-BI.

Cross-linking assay of apoA-I binding to ABCA1

The binding assays were conducted as described by Wang et al. (62) with minor modifications. HEK293-EBNA-T cells were plated as described above on 12-well poly-D-lysine-coated plates and 3 days later were transfected with plasmids when the cells were ~95% confluent, as described above. On day 4, the cells were preincubated in DMEM (high glucose) and 0.2% (w/v) fatty acid-free BSA with the indicated amounts of compounds at 37°C for 1 h before incubation at 37°C for 1 h with 2 μg/ml ^{125}I-apoA-I and the indicated compounds in the presence or absence of a 30-fold excess of unlabeled apoA-I. Cells were then washed, reversibly cross-linked with DSP (2 ml/well), and washed again as previously described (62). For immunoprecipitation, cell lysates prepared in buffer RI [50 mM Tris, pH 7.6, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40] containing a protease inhibitors mixture (Roche Molecular Biochemicals GmbH), and 1 mM phenylmethylsulfonyl fluoride were subjected to centrifugation at 1,000 *g* in a microcentrifuge for 10 min. The supernatant was collected and precleared with 10 μl of GammaBind G Sepharose beads for 1 h at room temperature with rotation. Polyclonal anti-ABCA1 antibody (50 μg) directed against the C terminus of ABCA1 (61) was added to the preabsorbed cell lysates and incubated with rotation at room temperature for 2 h. Twenty microliters of GammaBind G Sepharose beads was then added, and the incubation with rotation was continued at room temperature for 1 h. The samples were subjected to a brief centrifugation, and the pellets were washed three times with cold buffer RI at room temperature. The bound proteins were eluted from the beads by incubation and boiling in Laemmli sample buffer in the presence of 5% β-mercaptoethanol, which also serves to break the DSP-mediated cross-links between apoA-I and ABCA1. The eluted proteins were fractionated by 12% SDS-polyacrylamide gel electrophoresis, and the radioactivity in the dried gels was analyzed using a PhosphorImaging STORM860 system (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant software.

Determination of ABCA1 expression levels

To determine the effect of the compounds on the cell surface expression of ABCA1, we used previously described quantitative assays for cell surface ABCA1 that detects a FLAG tag inserted in the first large extracellular loop of ABCA1 (27). In brief, HEK293-EBNA-T cells on 24-well plates were transfected with a FLAG-ABCA1 cDNA or empty vectors as described above. The media containing DNA and lipofectamine were removed 1 day after transfection, and 1 day later the cells were exposed for 5 h at 37°C to assay medium [DMEM (high glucose) supplemented with 0.2% fatty acid-free BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4] with or without 150 μM BLT-4 or 500 μM glyburide. The cells were then chilled on ice for 10 min, and cell surface ABCA1-FLAG expression was measured as described by Fitzgerald et al. (27).

Total cellular ABCA1 expression was measured by immunoblot analysis using a polyclonal antibody as previously described (61). Transfected cells were treated for 5 h at 37°C with 500 μM glyburide or 150 μM BLT-4 in assay medium [DMEM (high glucose) supplemented with 0.2% fatty acid-free BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4] and subsequently lysed in a hypotonic buffer (250 mM sucrose, 10 mM HEPES, pH 7.4, and 1 mM EDTA) supplemented with a protease inhibitor mini-cocktail containing EDTA (Roche). Nuclei and cell debris were removed by centrifugation (800 *g* for 10 min). The amount of protein was measured in the postnuclear supernatants by the method of Bradford (63) and 15 μg of each sample were separated by 6% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were processed for antibody staining, and ABCA1 was detected using an enhanced chemiluminescence system (Pierce) as previously described (61).

Measurement of total cellular cholesterol and cholesteryl ester levels by TLC

HEK, ldlA7, and ldlA[mSR-BI] cells were labeled with [^{3}H]cholesterol as described above for cholesterol efflux assays. After incubation for 5 h with or without 500 μM glyburide or 150 μM BLT-4, the cells were lysed (lysis buffer: 50 mM Tris, pH 7.6, 150 mM NaCl, 0.25% sodium deoxycholate, and 1% Nonidet P-40) for 30 min at room temperature. A mixture of 2:1 chloroform-methanol (v/v) containing unlabeled lipids (200 μg/μl cholesterol and 200 μg/μl cholesteryl oleate) was added to the lysate as an internal standard. The organic phase of the resulting mixture was evaporated under nitrogen, dissolved in 75 μl of chloroform, and fractionated by TLC using ITLC SA plates (Pal Corporation) using petroleum ether-ether-acetic acid (85:15:1). Spots containing cholesteryl ester, cholesterol, and origin spots were cut out, and radioactivity was measured by liquid scintillation counting.

Synthesis of BLT-4

Naphthyl isocyanate (Sigma-Aldrich) was dissolved in ethyl acetate and *N*-methyl-pyrrolidinone (Sigma-Aldrich) and then cooled to 0°C. 2-Anisidine was added drop-wise by syringe over 10 min. Solid naphthyl isocyanate (1.00 g, 5.92 mmol) was dissolved in 45 ml of anhydrous 1:2 *N*-methyl pyrrolidinone-ethyl acetate (Acros Organics) under a nitrogen atmosphere and cooled to 0°C. 2-Anisidine (900 μl, 7.32 mmol) was added dropwise by syringe over 10 min and the reaction was stirred, first at 0°C for 2 h and then for an additional 2 h at room temperature. The reaction was quenched by the addition of 100 ml of 0.1 N HCl and extracted three times with 50 ml of dichloromethane. The pooled organic fractions were dried over anhydrous potassium carbonate, concentrated by rotary evaporation, and purified by flash chromatography using silica gel 60 (EMD Pharmaceuticals) equilibrated in 2:1 (v/v) hexane-ethyl acetate. BLT-4 was eluted with 1:1 (v/v) hexane-ethyl acetate and lyophilized with a final yield of 22%. The product was more than 95% pure as determined by liquid chromatography-mass spectrometry and NMR spectroscopy. The ^{1}H-NMR resonance frequencies for BLT-4 are as follows: (d_6-Me_2SO): δ 3.89 (3H, s), 6.90 (1H, td, J = 1.5 Hz, 1.5 Hz), 6.96 (1H, td, J = 1.5, 5.5 Hz), 7.02 (1H, dd, J = 7, 1.5 Hz), 7.34 (1H, T, J = 8 Hz), 7.44 (3H, m), 7.77 (1H, s), 7.79 (1H, s), 7.81 (1H, d, J = 4 Hz), 7.84 (1H, s), 8.12 (1H, d, J = 2 Hz), 8.17 (1H, dd, J = 1.5, 6.5 Hz), 8.30 (1H, s), 9.52 (1H, s).

RESULTS AND DISCUSSION

Effects of BLTs on ABCA1-mediated [^{3}H]cholesterol efflux to apoA-I

To determine if SR-BI- and ABCA1-mediated cholesterol efflux share common features, we examined the effects of BLTs on ABCA1-mediated cholesterol efflux from HEK293 cells transiently transfected with either an ABCA1-expression vector (HEK[ABCA1]) or an empty vector control (HEK[control]).

Cholesterol efflux was measured from cells labeled with unesterified [^{3}H]cholesterol for 24 h, followed by a 1 h preincubation with the BLT. This was followed by a 4 h incubation with the BLT in the presence or absence of 10 μg of protein per milliliter of lipid-free apoA-I, which served as an acceptor for cholesterol efflux. The amounts of [^{3}H]cholesterol in the incubation media and the amounts remaining associated with the cells were measured, and efflux was expressed as the percentage of cellular [^{3}H]cholesterol released into the medium during the 4 h incubation. **Figure 2** shows that the ABCA1-mediated [^{3}H]cholesterol efflux to apoA-I (closed bars) was not in-

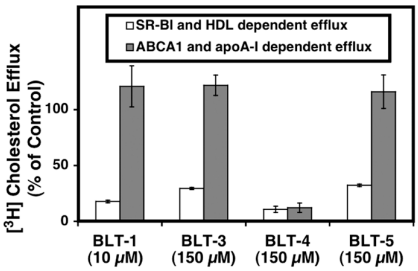

Fig. 2. Effects of BLTs on ABCA1- and scavenger receptor class B type I (SR-BI)-dependent efflux of [^{3}H]cholesterol to extracellular acceptors. HEK293-EBNA-T cells were transiently transfected with either an ABCA1 expression vector or the control pcDNA1 vector. One day after transfection, the cells were labeled for 24 h with [^{3}H]cholesterol and then preincubated for 1 h at 37°C in assay medium containing 0.5% (v/v) DMSO with or without the indicated concentrations of BLTs. After the preincubation, the cells were incubated for an additional 4 h at 37°C with the same concentrations of BLTs in the presence or absence of 10 μg/ml lipid-free recombinant human apolipoprotein A-I (apoA-I) to measure cholesterol efflux. The amounts of [^{3}H]cholesterol transferred from the cells to the medium and the values for ABCA1- and apoA-I-dependent efflux were determined as described in Experimental Procedures (closed bars). The control efflux values (percentage of cellular cholesterol released into the medium) measured in the absence of BLTs were as follows: HEK[ABCA1] with apoA-I, 7.4%; HEK [ABCA1] without apoA-I, 4%; HEK[control] with apoA-I, 1.7%; HEK[control] without apoA-I, 1%. The 100% control value determined in the absence of BLTs for ABCA1- and apoA-I-dependent efflux was 2.7% [calculated as (7.4-4) − (1.7-1)%]. Error bars represent the range of duplicate determinations. In independent experiments, SR-BI-mediated and HDL-dependent [^{3}H]cholesterol efflux was determined in [^{3}H]cholesterol-labeled ldlA[mSR-BI] cells (open bars). Cells were preincubated with the indicated amounts of BLTs for 1 h and then incubated for 2 h at 37°C with 500 μg of protein per milliliter of HDL and the same concentrations of BLTs in the presence or absence of a 1:800 dilution of the anti-SR-BI blocking antibody KKB-1 (25). The 100% of control value represents efflux in the absence of compound (50% of initial cellular radiolabeled cholesterol for efflux to HDL), and the 0% of control value represents efflux in the presence of HDL and the KKB-1 antibody (1:800 dilution) (12% of initial cellular radiolabeled cholesterol). There was no effect of the compounds on efflux to HDL in the presence of KKB-1. Error bars represent standard deviations from triplicate measurements.

hibited by BLT-1 (10 μM), BLT-3 (150 μM), or BLT-5 (150 μM), at concentrations that inhibit SR-BI-mediated [^{3}H] cholesterol efflux to HDL from ldlA[mSR-BI] cells, a cell line stably transfected with murine SR-BI (open bars) (45). At these concentrations, all three BLTs inhibited cholesterol efflux to HDL from HEK cells transfected with a cDNA construct encoding murine SR-BI, indicating that HEK cells are not intrinsically resistant to BLT-1, BLT-3, and BLT-5 (data not shown). Because the structures of BLT-1 and BLT-2 are very similar (45), we did not test BLT-2 in these studies. In contrast to the other BLTs, BLT-4 (Fig. 1B) at a concentration of 150 μM inhibited ABCA1- and apoA-I-dependent efflux by ~90%, approximately the same extent of its inhibition of cholesterol efflux observed for SR-BI-mediated efflux to HDL (Fig. 2) from transfected ldlA-7 (45) or HEK293 (data not shown) cells.

Figure 3A shows the concentration dependence of BLT-4-mediated inhibition of [^{3}H]cholesterol efflux. HEK [ABCA1] and HEK[control] cells were labeled with [^{3}H] cholesterol and incubated with or without lipid-free apoA-I and the indicated concentrations of BLT-4. As described previously (28, 29, 44, 61, 64), we found that [^{3}H]cholesterol efflux depended on the expression of ABCA1 and

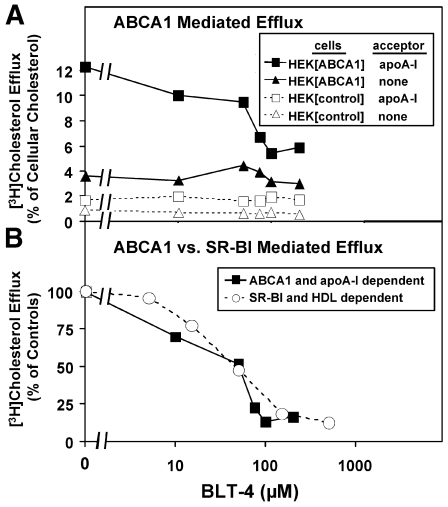

Fig. 3. Concentration dependence of the inhibition of ABCA1- and SR-BI-mediated [^{3}H]cholesterol efflux by BLT-4. A: [^{3}H]cholesterol efflux from HEK[ABCA1] (closed symbols) or HEK[control] (open symbols) cells was determined in the presence (squares) or absence (triangles) of 10 μg/ml lipid-free recombinant human apoA-I and the indicated concentrations of BLT-4 as described in the legend to Fig. 2 and Experimental Procedures. Values are expressed as the percentage of cellular [^{3}H]cholesterol released into the medium after a 4 h incubation at 37°C. B: The effect of BLT-4 on ABCA1-mediated and apoA-I-dependent [^{3}H]cholesterol efflux (closed squares) was calculated from the data in A as described in the legend to Fig. 2 and Experimental Procedures. In an independent experiment, SR-BI-mediated and HDL (500 μg protein/ml)-dependent [^{3}H]cholesterol efflux (open circles) was determined as described in the legend to Fig. 2 and Experimental Procedures. The maximum efflux in the absence of BLT-4 (60% of total cellular cholesterol) was set to 100% (the 0% of control was 19%).

the presence of apoA-I as an acceptor in the incubation medium. As the concentration of BLT-4 increased, there was a substantial inhibition of efflux from HEK[ABCA1] cells incubated with apoA-I, but there was little or no inhibition of the relatively low background efflux from HEK[ABCA1] cells in the absence of apoA-I or from HEK[control] cells either with or without apoA-I. Figure 3B shows that the ABCA1- and apoA-I-dependent [^{3}H] cholesterol efflux, calculated as described in Experimental Procedures, was inhibited by BLT-4 with an IC_{50} of ~60 μM. This value is similar to that for its inhibition of SR-BI-dependent [^{3}H]cholesterol efflux to HDL (IC_{50} ~ 55 μM; Fig. 3B) (45).

The shared sensitivities to BLT-4 suggest that there may be similarities in the mechanisms by which BLT-4 inhibited ABCA1- and SR-BI-mediated cellular cholesterol efflux. The inability of BLT-1, BLT-3, and BLT-5 to inhibit ABCA1 raises the possibility that the mechanisms by which they block SR-BI activity may differ from that of BLT-4, although additional studies will be required to directly address this question.

Effects of the ABCA1 inhibitor glyburide on SR-BI-mediated [^{3}H]cholesterol efflux to HDL

The inhibition of ABCA1-mediated cholesterol efflux by BLT-4 prompted us to determine if the ABCA1 inhibitor glyburide (29, 31, 46–51) (Fig. 1A) could block SR-BI-mediated [^{3}H]cholesterol efflux to HDL. **Figure 4** compares the concentration dependence of glyburide's inhibition of ABCA1-mediated [^{3}H]cholesterol efflux to apoA-I (A, C) with its effects on SR-BI-mediated [^{3}H]cholesterol efflux to HDL (B, C).

As expected from previous studies (29, 31, 48–50), glyburide inhibited ABCA1-mediated [^{3}H]cholesterol efflux from HEK[ABCA1] cells to apoA-I (10 μg protein/ml) with an IC_{50} of ~300 μM (Fig. 4A), and it had little effect on the low background efflux in the absence of ABCA1 transgene expression, in the absence of apoA-I, or both. This IC_{50} value is substantially greater than that for BLT-4 (~60 μM). Thus, BLT-4 was a more potent inhibitor of ABCA1 than was glyburide.

Strikingly, glyburide's effects on SR-BI-mediated efflux were virtually identical to those on ABCA1-mediated efflux. Figure 4B shows that glyburide inhibited SR-BI-mediated [^{3}H]cholesterol efflux from ldlA[mSR-BI] cells to HDL (500 μg protein/ml) with a IC_{50} of ~275 μM, and it had little effect on the low background efflux in the absence of SR-BI transgene expression (ldlA-7 control cells), in the absence of HDL, or both. Figure 4C, which compares directly glyburide's inhibition of ABCA1- and apoA-I-dependent cholesterol efflux and its inhibition of SR-BI- and HDL-dependent cholesterol efflux, shows that the drug is equally potent in inhibiting these cell surface transport proteins.

We also found that lipid-free apoA-I (10 μg protein/ml) was a poor acceptor of SR-BI-mediated [^{3}H]cholesterol efflux compared with HDL (500 μg protein/ml) [2% vs. 61%, respectively; also see ref. (36)]. The low level of lipid-free apoA-I-dependent [^{3}H]cholesterol efflux from

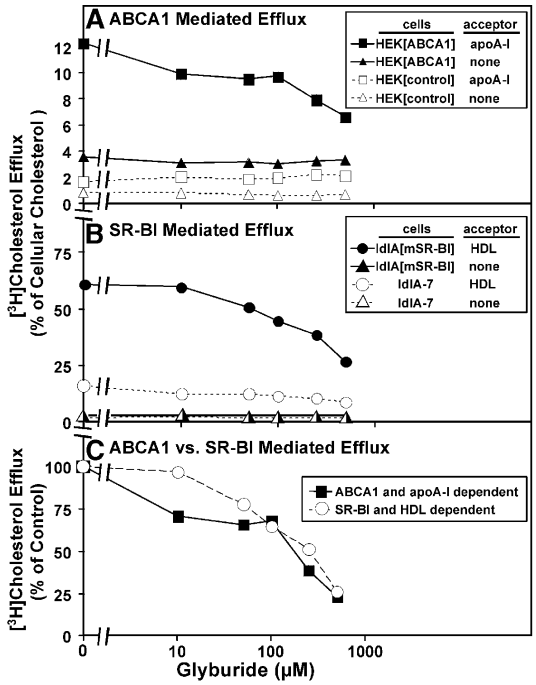

Fig. 4. Concentration dependence of the inhibition of ABCA1- and SR-BI-mediated [^{3}H]cholesterol efflux by glyburide. A: [^{3}H]cholesterol efflux from HEK[ABCA1] (closed symbols) or HEK[control] (open symbols) cells was determined in the presence (squares) or absence (triangles) of 10 μg/ml lipid-free recombinant human apoA-I and the indicated concentrations of glyburide as described in Experimental Procedures. B: [^{3}H]cholesterol efflux from SR-BI-expressing ldlA[mSR-BI] (closed symbols) or control ldlA-7 (open symbols) cells was determined in the presence (circles) or absence (triangles) of 500 μg of protein per milliliter of HDL and the indicated concentrations of glyburide as described in the legend to Fig. 3B. C: ABCA1-mediated and apoA-I-dependent [^{3}H]cholesterol efflux (closed squares) was calculated from the data in A as described in Experimental Procedures. SR-BI-mediated and HDL-dependent [^{3}H]cholesterol efflux (open circles) was calculated from the data in B. Efflux to HDL in ldlA[mSR-BI] cells in the absence of compound, and in ldlA-7 cells in the presence of compound, was used to set the 100% and 0% of control values, respectively. There was a small, KKB-1-inhibitable effect of glyburide on cholesterol efflux to HDL in ldlA-7 cells, which represents the efflux mediated by the low levels of endogenous SR-BI that is expressed in these cells.

ldlA[mSR-BI] and untransfected ldlA-7 control cells (∼2%) was insensitive to glyburide (data not shown). It is thus unlikely that glyburide's ability to affect SR-BI's activity has complicated the interpretation of previous studies of ABCA1 activity, because those studies used lipid-free apoA-I as an acceptor of glyburide-sensitive cholesterol efflux.

We and others have found that the concentration of glyburide required to inhibit ABCA1 is substantially higher than its reported in vitro Ki for other ABC proteins, such as SUR1/2 [reviewed in ref. (53)]. It is possible that the inhibition of ABCA1-mediated cholesterol efflux by glyburide is a secondary consequence of high concentrations of glyburide interfering with other cellular processes, including features of cellular cholesterol metabolism. We were unable to detect glyburide inhibition of cholesterol esterification (thin layer chromatographic assay) or of the total cellular cholesterol levels in either HEK cells or ldlA[mSR-BI] cells loaded with [^{3}H]cholesterol (data not shown). Thus, at least some aspects of cellular cholesterol metabolism appear to be unaffected by high-dose glyburide treatment under the conditions used in these experiments. We previously have shown that BLT-4 is a relatively specific inhibitor in that it does not affect multiple membrane transport processes, such as clathrin-dependent endocytosis, lipid-raft-dependent endocytosis, and the protein secretory pathway, nor does it have a detectable impact on the integrity of the actin and microtubular networks (45). As was the case with glyburide, BLT-4 did not appear to inhibit cholesterol esterification or to alter total cellular cholesterol levels in either HEK cells or ldlA[mSR-BI] cells loaded with [^{3}H]cholesterol (data not shown).

Effects of glyburide on SR-BI-mediated [^{3}H]COE uptake from [^{3}H]COE-HDL and ^{125}I-HDL binding

Previous studies have shown a close relationship between SR-BI-mediated binding to HDL with both SR-BI-mediated cholesterol efflux to HDL and selective lipid uptake from HDL (24, 57, 65–68). We tested the effects of glyburide on these two additional activities of SR-BI. **Figure 5** shows that glyburide suppressed SR-BI-mediated [^{3}H]COE uptake from [^{3}H]COE-HDL by ldlA[mSR-BI] cells ($IC_{50} \sim 150$ μM). Flow cytometric analysis of the surface expression of SR-BI established that the effects of glyburide (at concentrations of ⩽500 μM) on SR-BI's activities were not attributable to a reduction in the steady-state level of SR-BI on the cell surface (data not shown).[2] Thus, glyburide inhibited both lipid transport activities of SR-BI, selective uptake and cholesterol efflux, as is the case for the BLTs (45).[3]

BLTs 1–5 increase the affinity of ^{125}I-HDL binding to SR-BI at 37°C (lower apparent K_d) (45). The decreased apparent K_d values are attributable, at least in part, to decreased dissociation rates and are not accompanied by substantially altered maximal binding values. Figure 5 shows that increasing concentrations of glyburide in-

[2] When ldlA[mSR-BI] or ldlA7 cells were treated with a very high concentration of glyburide (1 mM) for a total of 3 h, we observed morphological changes (e.g., changes in the appearance of the nucleus) and occasionally signs of toxicity as measured by trypan blue exclusion.

[3] Glyburide at a concentration of 1 mM has been reported to prevent specific ABCA1- and apoA-I-dependent phospholipid (phosphatidylcholine) efflux from cells, determined as the difference in phospholipid efflux from ABCA1-expressing cells measured in the presence and absence of apoA-I in the extracellular medium (29). We also observed this inhibition at very high doses of glyburide (500 μM and higher). However, we found in our experiments that the glyburide-induced decrease in specific apoA-I-dependent phospholipid efflux was largely attributable to an increase in ABCA1-mediated efflux to the apoA-I-free medium rather than to a substantial decrease in efflux to the apoA-I containing medium (data not shown).

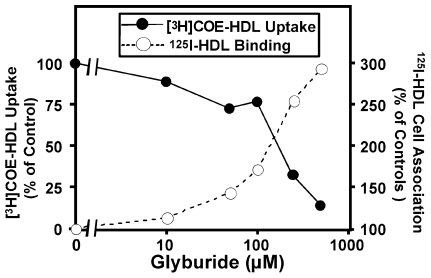

Fig. 5. Effects of glyburide on SR-BI-mediated [^{3}H]cholesteryl oleyl ether ([^{3}H]COE) uptake from [^{3}H]COE-HDL and ^{125}I-HDL binding. SR-BI-expressing ldlA[mSR-BI] or control ldlA-7 cells were preincubated with the indicated concentrations of glyburide, and then the uptake of [^{3}H]COE from [^{3}H]COE-HDL (10 μg protein/ml) (closed circles) or the binding of ^{125}I-HDL (10 μg protein/ml) (open circles) was determined in the presence or absence of a 40-fold excess of unlabeled HDL (duplicate incubations). The specific values (difference between the values in the absence and presence of excess HDL) were normalized such that the value in the absence of glyburide was set to 100%. The 100% of control values (nanograms of HDL protein per milligram of cell protein) for ldlA[mSR-BI] cells were as follows: [^{3}H]COE uptake, 1,962; ^{125}I-HDL binding, 198. The 100% of control values for ldlA-7 cells were as follows: [^{3}H]COE uptake, 289; ^{125}I-HDL binding, 21.

creased the binding of ^{125}I-HDL (10 μg protein/ml) to ldlA[mSR-BI] cells in a manner that was correlated inversely with its inhibition of lipid transport (Figs. 4 and 5). The increased binding of ^{125}I-HDL to SR-BI observed at a subsaturating concentration of ^{125}I-HDL appeared to be attributable, at least in part, to a glyburide-induced increase in the affinity of SR-BI for ^{125}I-HDL without a substantial change in the maximal binding values (**Fig. 6**). For the ^{125}I-HDL concentrations used in these experiments [a relatively broad density range (23)], the apparent K_d values were 26.2 ± 3.7 μg protein/ml in the absence of glyburide and 4.7 ± 0.7 μg protein/ml in the presence of 250 μM glyburide. Thus, the effects of the ABCA1 inhibitor glyburide on SR-BI's activities were similar to the activities of the BLTs: increased HDL binding and decreased lipid transport.

Effects of BLT-4 and glyburide on apoA-I binding to ABCA1

The increase in affinity of SR-BI for HDL induced by BLT-4 [see also ref. (45)] and glyburide prompted us to examine the binding of apoA-I to ABCA1. **Figure 7** shows a representative assay that involved incubating ABCA1-expressing cells with ^{125}I-apoA-I, treating the cells with a cleavable cross-linking agent (DSP), immunoprecipitating cross-linked complexes with anti-ABCA1 antiserum, and then detecting the coprecipitated ^{125}I-apoA-I after breaking the cross-links and fractionating the samples by SDS-PAGE (62). As expected, there was no detectable binding of ^{125}I-apoA-I to control cells transfected with empty vector (no ABCA1). Both glyburide [250–1,000 μM; see also ref. (62)] and BLT-4 (150 μM) effectively blocked the binding of ^{125}I-apoA-I to ABCA1 at concentrations that inhibit ABCA1-mediated cholesterol efflux to apoA-I (Figs.

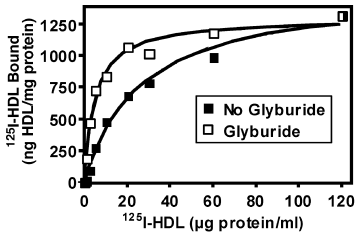

Fig. 6. Effects of glyburide on the concentration dependence of ^{125}I-HDL binding to SR-BI-expressing cells. SR-BI-expressing ldlA[mSR-BI] cells were preincubated in the absence (closed squares) or presence (open squares) of 250 μM glyburide and then incubated for 2 h at 37°C with the same amount of glyburide and the indicated concentrations of ^{125}I-HDL in the presence (single incubations) or absence (duplicate incubations) of a 40-fold excess of unlabeled HDL. The specific binding values are shown.

2–4). We did not detect a decrease in either the cell surface levels or the total protein levels of ABCA1 in cells treated under similar conditions with either compound (data not shown). Thus, glyburide and BLT-4 inhibition of ^{125}I-apoA-I binding to ABCA1 cannot be explained by decreases in either cell surface or total levels of ABCA1. The inhibition of apoA-I binding to ABCA1 by glyburide and BLT-4 contrasts their abilities to enhance the affinity of HDL binding to SR-BI.

It is possible that the reciprocal inhibition of SR-BI and ABCA1 by the structurally distinct small molecules BLT-4 and glyburide was simply a coincidence of multiple independent activities of these compounds. An alternative, more appealing explanation is that, despite the very different structures and physiologic functions of SR-BI and

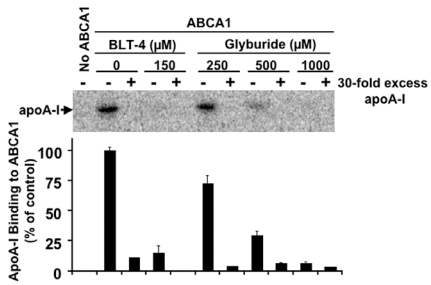

Fig. 7. Effects of BLT-4 and glyburide on ^{125}I-apoA-I binding to ABCA1. HEK293-EBNA-T cells were transiently transfected with either an ABCA1 expression vector (ABCA1) or a control pcDNA1 vector (No ABCA1). One day after transfection, the cells were preincubated for 1 h at 37°C in assay medium with the indicated concentrations of BLT-4 or glyburide. The binding of ^{125}I-apoA-I (2 μg/ml, 1 h, 37°C) in the presence or absence of a 30-fold excess of unlabeled apoA-I and the indicated concentrations of inhibitors was measured using a dithiobis (succinimidyl propionate) cross-linking assay as described in Experimental Methods. Bound ^{125}I-apoA-I was fractionated by SDS-PAGE and detected in the gels using a PhosphorImaging STORM860 system (top), and the relative amounts of ^{125}I-apoA-I in the lanes were determined using ImageQuant software (bottom). Values shown in the lower portion of the figure are representative of two independent experiments performed in duplicate. Error bars represent the ranges of duplicate determinations.

ABCA1, some features of their mechanisms of lipid transport may have similarities or even share common steps that are sensitive to BLT-4 and glyburide. This explanation is supported by the observation that the potencies (IC_{50}) for glyburide inhibition of SR-BI and ABCA1 are virtually identical, as they are for BLT-4.

The molecular targets of BLT-4 and glyburide responsible for their inhibition of SR-BI- and ABCA1-mediated lipid transport activities have not yet been identified, and they need not be the same. However, these lipid transport proteins themselves are obvious candidates. Glyburide and BLT-4 might bind to either the same or distinct sites on each protein. It is also possible that the direct targets of these inhibitors are not the transporters themselves but rather some other protein(s) or lipids (e.g., a specific membrane domain). It is noteworthy that, even though the structures of glyburide and BLT-4 (with the exception of the methoxyphenyl and the urea functionalities) differ (Fig. 1), they both can inhibit the activities of several ABC proteins. BLT-4 inhibits ethidium bromide efflux from *Staphylococcus aureus* mediated by NorA, a member of the ABC superfamily (69), as well as ABCA1 activity (this study). Glyburide, which was originally identified as a drug that binds to and inhibits the ABC subunit (SUR) of the ATP-sensitive K^+_{ATP} channels, can interfere with pathways involving the ABC proteins cystic fibrosis transmembrane regulator (54), bile salt export pump (56), and multidrug resistance P-glycoprotein (55), as well as ABCA1 (29, 31, 46–51). It is possible that the target of the inhibitory activities of both drugs could be a cell surface or intracellular ABC transporter(s) other than ABCA1 that may directly or indirectly influence lipid transport mechanisms mediated by both SR-BI and ABCA1.

Despite the similar abilities of either glyburide or BLT-4 to inhibit lipid transport mediated by ABCA1 and SR-BI, there are several striking differences in the mechanisms by which these transporters function. Lipid-free apoA-I binds preferentially to ABCA1, and along with preβ-apoA-I it is the preferred cholesterol acceptor from ABCA1 relative to spherical, lipid-rich HDL particles (32–36). In contrast, spherical, lipid-rich HDL particles are the preferred ligands for SR-BI (15). The effects of glyburide and BLT-4 on ligand binding also highlight the differences between these transporters. Both inhibitors block ligand binding to ABCA1, whereas they enhance the ligand binding affinity of SR-BI. Additional studies will be required to elucidate the mechanisms underlying the complex effects of these inhibitors on these key cholesterol transporters.

In summary, we have identified BLT-4 as a new and relatively potent inhibitor of ABCA1. We also show that glyburide is a heretofore unrecognized inhibitor of SR-BI. The reciprocal inhibition of these transport proteins by BLT-4 and glyburide raises the possibility that the mechanisms of lipid transport by SR-BI and ABCA1 might possibly involve another ABC transporter.

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CHAPTER IV

Endocytosis is not required for the selective lipid uptake mediated by murine SR-BI

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Endocytosis is not required for the selective lipid uptake mediated by murine SR-BI

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Abstract

The scavenger receptor class B, type I (SR-BI) mediates the cellular selective uptake of cholesteryl esters and other lipids from high-density lipoproteins (HDL) and low-density lipoproteins (LDL). This process, unlike classical receptor-mediated endocytosis, does not result in lipoprotein degradation. Instead, the lipid depleted particles are released into the medium. Here we show that selective lipid uptake mediated by murine SR-BI can be uncoupled from the endocytosis of HDL or LDL particles. We found that blocking selective lipid uptake by incubating cells with the small chemical inhibitors BLT-1 or BLT-4 did not affect endocytosis of HDL. Similarly, blocking endocytosis by hyperosmotic sucrose or K^+ depletion did not prevent selective lipid uptake from HDL or LDL. These findings suggest that mSR-BI-mediated selective uptake occurs at the cell surface upon the association of lipoproteins with mSR-BI and does not require endocytosis of HDL or LDL particles.

Keywords: High-density lipoprotein; Low-density lipoprotein; Scavenger receptor; Scavenger receptor class B, type I; Endocytosis; BLT

1. Introduction

The high-density lipoprotein (HDL) receptor SR-BI (scavenger receptor class B, type I) plays an important role in the metabolic control of HDL [1]. SR-BI mediates the selective uptake of cholesteryl esters [2] and other lipids [3–6] from HDL particles by cells, and it also facilitates the transfer of cholesterol from cells to HDL or to other acceptors present in the extracellular environment [7–9]. In addition, SR-BI binds to [10] and mediates selective lipid uptake from LDL [11–14], although to a lesser extent than from HDL. Results from in vitro experiments indicate that liposomes containing SR-BI and no other proteins are sufficient to sustain the selective transfer of cholesteryl esters from HDL particles to these liposomes [15].

Intracellular lipoprotein degradation is not required for selective lipid uptake in cells expressing SR-BI, because subsequent to lipid depletion most or all the HDL [2,16–18] or LDL particles [19–21] are released to the extracellular space. Although this is consistent with lipid transfer occurring at the cell surface, it does not rule out the possibility that lipid transfer might occur in a specialized endosomal compartment and thus depend on the internalization of HDL or LDL complexed to SR-BI. The model of endocytosis-independent selective lipid uptake is supported by the in vitro liposome reconstitution experiments [15] and electron microscopic images showing little or no intracellular HDL in cultured murine adrenal cells [22] or in steroidogenic tissues [23] that naturally exhibit high levels of SR-BI-mediated selective uptake. It is also supported by the observation that the transfer of fluorescently labeled-cholesterol from HDL to cells expressing SR-BI was not affected by ATP depletion, which blocked HDL internalization [24]. The alternative model of endocytosis-depend-

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ent selective lipid uptake is supported by reports showing that the extent of HDL cycling between the cell surface and intracellular compartments is directly correlated with the efficacy of selective uptake [25–27].

To distinguish between these two models, we took advantage of distinct treatments that prevent either cellular endocytosis or SR-BI-mediated selective lipid transport to ask if the blockage of one pathway affects the other. We found that exposure of cells expressing murine SR-BI (mSR-BI) to BLT-1 or BLT-4, two potent small chemical inhibitors of SR-BI-dependent selective lipid uptake [28], did not interfere with the low level of internalization of HDL mediated by mSR-BI. The transient treatment of cells to block endocytosis with hyperosmotic sucrose [29,30] or the depletion of intracellular potassium [30,31] prevented the low levels of mSR-BI-mediated internalization of HDL and LDL, but did not inhibit mSR-BI-mediated selective uptake from these lipoproteins. Taken together, these results show that lipoprotein internalization is not a requisite for efficient mSR-BI-mediated lipid transport.

2. Materials and methods

2.1. Chemicals

Stock solutions of BLT-1 (Chembridge Corp.) and BLT-4 [32] were prepared in 100% DMSO and diluted into the appropriate assay media immediately prior to use. The final concentration of DMSO in the media was 0.5% (v/v). The control assay media contained 0.5% DMSO without BLT.

2.2. Lipoproteins

Human HDL and LDL were isolated and labeled with Alexa Fluor 568 (Molecular Probes) (Alexa-HDL and Alexa-LDL), ^{125}I (^{125}I-HDL), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate (DiD, Molecular Probes) (DiD-HDL and DiD-LDL), and [^{3}H]cholesteryl oleyl ether ([^{3}H]CEt, [^{3}H]CEt-HDL and [^{3}H]CEt-LDL) as described [2,10,33–35]. LDL was labeled with [^{3}H]CEt according to the [^{3}H]CEt-HDL labeling procedure [2].

2.3. Cells

LDL receptor deficient Chinese hamster ovary cells that express low levels of endogenous SR-BI (ldlA-7, [36]) and ldlA-7 cells that were stably transfected to express high levels of murine SR-BI (ldlA[mSR-BI]) [2] were grown in Ham's F12 culture medium supplemented with 5% (v/v) fetal bovine serum (FBS), 100 μg/ml each of penicillin and streptomycin and 500 μg/ml G418 (for ldlA[mSR-BI] cells). Y1-BS1 adrenal cells were grown in an RMPI culture medium (Gibco BRL) supplemented with 10% fetal bovine serum and 100 μg/ml each of penicillin and streptomycin. The expression of endogenous SR-BI was induced by treating the Y1-BS1 cells for 24 h in the same medium supplemented with 0.1 μM adrenocorticotropic hormone (ACTH, Sigma) [37].

2.4. General assay conditions

Prior to the assay, cells were grown for 2 days on glass coverslips (for microscopy experiments) or in regular 24-well plates (for assays using radiolabeled lipoproteins). On the day of the assay, cells were washed twice in the appropriate culture medium supplemented with 0.5% bovine serum albumin and 25 mM HEPES/KOH, pH 7.4 but without serum, antibiotics or G418 (assay medium). For experiments involving DiD-labeled lipoproteins, we used Leibowitz medium (Gibco BRL) containing 0.5% bovine serum albumin (BSA) and 25 mM HEPES/KOH, pH 7.4. For control experiments, we used assay medium supplemented 0.5% DMSO.

2.5. Hypertonic sucrose treatment

Endocytosis was blocked by incubation with hypertonic sucrose [29,30]. Briefly, cells were incubated at 37 °C for 30 min with 0.45 M sucrose in the appropriate assay medium. Alexa-568, DiD, ^{125}I- or [^{3}H]CEt-labeled HDL or LDL was then added for an additional incubation of 1 h at the same temperature. During the last 20 min of the incubation, 50 μg/ml of Alexa488-labeled human holotransferrin (Molecular Probes) were added.

2.6. Potassium depletion

Endocytosis was blocked by potassium depletion [30,31]. Briefly, cells were washed twice with PBS/1 mM $MgCl_2$/0.1 mM $CaCl_2$ (PBS+) followed by one wash with hypotonic medium (PBS+/H_2O (1:3 v/v)) followed by a brief 5–7 min incubation with the hypotonic medium. The cells were then washed 5–8 times in a medium containing 150 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/20 mM HEPES/KOH pH 7.4/0.5% DMSO/0.5% BSA followed by a 30 min incubation in the same medium. Alexa-568, DiD or ^{125}I-labeled HDL or LDL was then added for an additional incubation of 1 h at the same temperature. During the last 20 min of the incubation, 50 μg/ml of Alexa488-labeled human holotransferrin (Molecular Probes) was added.

2.7. Preparation of cells for fluorescence microscopy

After exposure to Alexa-HDL or -LDL, cells were chilled on ice for 10 min, washed three times with PBS+ and then fixed for 1 h on ice with 4% (w/v) paraformaldehyde (PFA) dissolved in PBS+(PBS-PFA). The PFA was quenched by the addition of 50 mM NH_4Cl for 5 min at room temperature (RT). Following a wash with PBS+, the surfaces of the cells

were stained at RT for 30 min with Alexa647-labeled Concanavalin A (100 μg/ml, Molecular Probes) in PBS+ containing 5% (w/v) BSA (PBS-BSA). The cells were then washed in PBS-BSA, fixed again in PBS-PFA for 15 min at RT and quenched as described above. We used the lipophylic dye DiD instead of the more commonly used dye DiI to avoid the excitation of DiI by the 488 nm spectral laser line required to excite Alexa-488. After the exposure of cells to DiD-labeled HDL or LDL, the medium containing the fluorescent lipoproteins was removed and each coverslip was placed in a live-cell imaging open perfusion chamber at 37 °C containing hypertonic sucrose or buffer A (for K^+ depletion) to maintain the endocytic block. Cells were imaged with a spinning disk confocal microscope under the control of SlideBook 4 (Intelligent Imaging Innovations). Three-dimensional image stacks were recorded by sequential acquisition of optical sections along the *z*-axis with steps of 0.25 μm.

2.8. Cellular binding, uptake, and degradation of radiolabeled HDL and LDL

Assays of the uptake of [^{3}H]CEt from [^{3}H]CEt-HDL or [^{3}H]CEt-LDL, and the cellular degradation and binding of ^{125}I-HDL and ^{125}I-LDL (all at 10 μg protein/ml) were performed in ldlA[mSR-BI] cells and control untransfected ldlA-7 cells as described [2]. The amounts of cell-associated [^{3}H]cholesteryl ether are expressed as the corresponding amounts of protein (ng) in the [^{3}H]CEt-labeled lipoprotein to permit a direct comparison of the relative amounts of ^{125}I-HDL or ^{125}I-LDL binding and [^{3}H]CEt uptake [38]. Statistical analysis was performed with Graphpad Prism 3 software from Graphpad Software, Inc. (San Diego).

2.9. Flow cytometric analysis of mSR-BI surface expression

The surface expression of mSR-BI in ldlA[mSR-BI] and ldlA7 cells was determined using the polyclonal anti-SR-BI antibody KKB1 [33] as described [28].

3. Results

3.1. The inhibition of mSR-BI-mediated selective lipid uptake by BLTs does not prevent the endocytosis of HDL

It was previously shown that the expression of SR-BI results in the specific binding of HDL to the cell surface [2] and in the internalization of relatively little HDL [25–27,39]. These observations were confirmed by experiments using three-dimensional confocal fluorescence microscopy. In these experiments, stably transfected cells expressing high levels of mSR-BI (ldlA[mSR-BI] cells, [2]) were exposed for 1 h at 37 °C to HDL fluorescently labeled on its protein moiety with Alexa-568 (Alexa-HDL). Control untransfected ldlA-7 cells that express very little mSR-BI [36] exhibited little binding or endocytosis of the fluorescently labeled lipoprotein (Fig. 1A, circumscribed areas demarcate the inside of the cell). The colocalization of Alexa-HDL with the fluorescently labeled plasma membrane marker Concanavalin A demonstrated that in ldlA[mSR-BI] cells the majority of the HDL was bound at the cell surface (Fig. 1D, F). Only a small portion of the Alexa-HDL was found in endocytic compartments, visualized by colocalization with Alexa 488-transferrin internalized by receptor-mediated endocytosis (Fig. 1E) [40].

We have previously identified a set of small molecule inhibitors named BLTs that block SR-BI-mediated selective

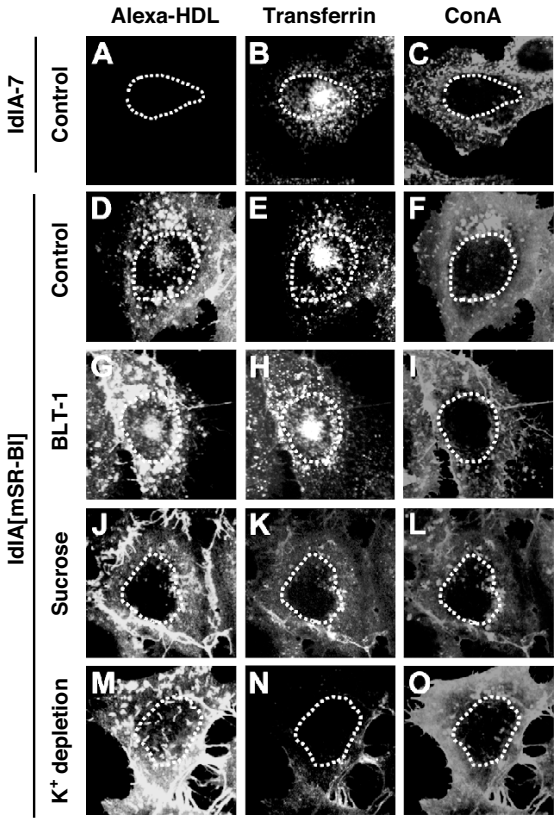

Fig. 1. Effects of BLT-1, hypertonic sucrose treatment and cellular potassium depletion on mSR-BI-mediated endocytosis of HDL. ldlA[mSR-BI] cells and ldlA-7 cells were incubated for 30 min with the control medium (A–F), 1 μM BLT-1 (G–I), hypertonic sucrose medium (J–L) or medium lacking potassium (M–O). The cells were then incubated for another 60 min with Alexa568-HDL (10 μg protein/ml) followed by the addition of Alexa488-transferrin (TF) (50 μg/ml) during the last 20 min of incubation. All the incubations were performed at 37 °C. Cells were transferred to 4 °C, fixed and incubated with Alexa647-Concanavalin A (ConA) to label the external cell surfaces. The cells were imaged using three-dimensional fluorescence confocal microscopy and representative confocal sections are shown. The white lines circumscribe the cell interior. Similar results were obtained using unlabeled HDL visualized with the polyclonal antibody 5F6 (specific for the major HDL protein apoA-I, University of Ottawa Heart Institute) in PFA fixed cells permeabilized with 0.1% saponin (not shown).

lipid uptake from HDL but do not block the entry of transferrin, EGFF or cholera toxin [28]. To test whether BLTs block selective uptake by preventing HDL internalization, we incubated the mSR-BI expressing cells with BLT-1, which is the most potent inhibitor. We found, as expected [28], a 83% decrease in the selective uptake of [^{3}H]cholesteryl ether ([^{3}H]CEt) from [^{3}H]CEt-HDL and a two-fold increase in cell-associated ^{125}I-HDL (not shown). Under these conditions in which selective lipid transfer was blocked, we observed an increase in the amount of Alexa-HDL bound at the cell surface, and a small increase in the amount of internalized Alexa-HDL (Fig. 1G). Similar results were obtained when cells were treated with 50 μM BLT-4 (not shown). These results suggest that the inhibition of selective lipid uptake by the BLTs is not a consequence of a block in the SR-BI-mediated endocytosis of the HDL particles.

3.2. The inhibition of HDL endocytosis does not prevent mSR-BI-mediated selective lipid uptake

The second approach we used to determine if lipoprotein internalization is required for mSR-BI-mediated selective lipid uptake was to examine the effects on selective lipid uptake of an acute block of endocytosis. A brief exposure of ldlA[mSR-BI] cells to hyperosmotic sucrose [29,30] or transient depletion of intracellular K^+ [30,31] results in the expected strong inhibition of receptor-mediated endocytosis of Alexa-488 transferrin (Fig. 1K, N, compared to B, E and H). Similarly, these perturbations interfered with the uptake of Alexa-HDL particles (Fig. 1J, M, compared to D and G). In contrast, the mSR-BI-dependent selective uptake of the fluorescent lipid probe DiD from DiD-labeled HDL particles to the cells was not affected (Fig. 2). Similar results (not shown) were obtained using ACTH-treated Y1-BS1 murine adrenal cortical cells [37] that normally express mSR-BI. Combined, these qualitative results suggest that blocking the internalization of HDL particles does not affect noticeably mSR-BI-mediated selective lipid uptake. Independent support for these observations was obtained by following the effect of hypertonic sucrose treatment on the cell association and degradation of ^{125}I-HDL, and on the selective uptake of [^{3}H]CEt from [^{3}H]CEt-HDL (Fig. 3A–C). These experiments showed that in ldlA[mSR-BI] cells under normal conditions most of the cell-associated ^{125}I-HDL, which represents both surface bound and internalized lipoprotein, was dependent on mSR-BI expression as there was little association in control ldlA-7 cells. Hypertonic sucrose treatment decreased this association by ~30% (Fig. 3A). In addition, the low level of intracellular ^{125}I-HDL proteolysis, as measured by the generation of ^{125}I-labeled acid soluble degradation products released in to the medium, was also reduced when the ldlA[mSR-BI] cells were treated with hyperosmotic sucrose (Fig. 3B). This was presumably due to the inhibition of HDL internalization, since the amount of HDL at the cell surface as determined by

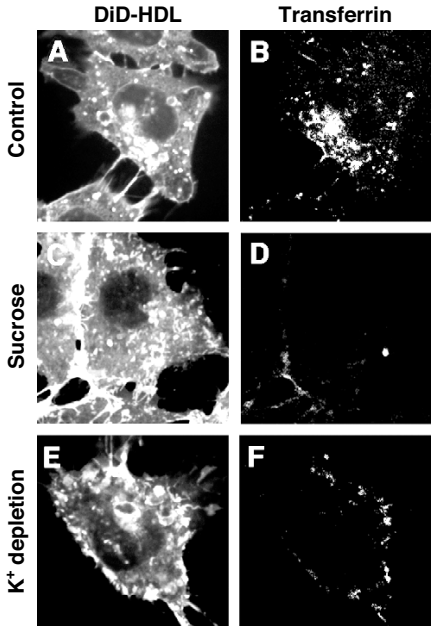

Fig. 2. Effect of hypertonic sucrose treatment and cellular potassium depletion on mSR-BI-mediated selective uptake of DiD from DiD-HDL. ldlA[mSR-BI] cells were incubated for 30 min with the medium only (A, B), hypertonic sucrose medium (C, D) or potassium-controlled medium (E, F) followed by the addition of DiD-labeled HDL (DiD-HDL) (80 μg protein/ml final) for another 60 min. Alexa488-transferrin (TF) was added during the last 20 min of incubation. All incubations were carried at 37 °C. Cells were washed at room temperature and imaged without fixation by confocal fluorescence microscopy. Representative sections are shown.

fluorescence microscopy of Alexa-HDL did not change (Fig. 1), and the level of SR-BI at the cell surface as was not altered (determined by flow cytometry, not shown). Taken together, these results demonstrate that hyperosmotic sucrose prevents the internalization of HDL mediated by mSR-BI. Under these conditions of endocytic blockade, however, the selective uptake of [^{3}H]CEt from [^{3}H]CEt-HDL was not affected (Fig. 3C). Thus, mSR-BI-mediated selective lipid uptake from HDL can occur independently of HDL endocytosis and degradation.

3.3. Uncoupling of mSR-BI-mediated LDL endocytosis and selective lipid uptake

Next, we asked if mSR-BI-mediated selective uptake from LDL requires endocytosis of LDL particles. As shown in Fig. 4A, there was virtually no surface binding or internalization of Alexa568-LDL by control ldlA-7 cells, which express essentially no SR-BI [2] or LDL receptor [36]. In contrast, the expression of mSR-BI in ldlA[mSR-BI] cells led to the intracellular accumulation of LDL labeled on its protein moiety with Alexa568 (Alexa-LDL) in an endosomal compartment (Fig. 4D), identified by the colocalization with Alexa488-transferrin internalized through the clathrin-mediated pathway (Fig. 4E). Further-

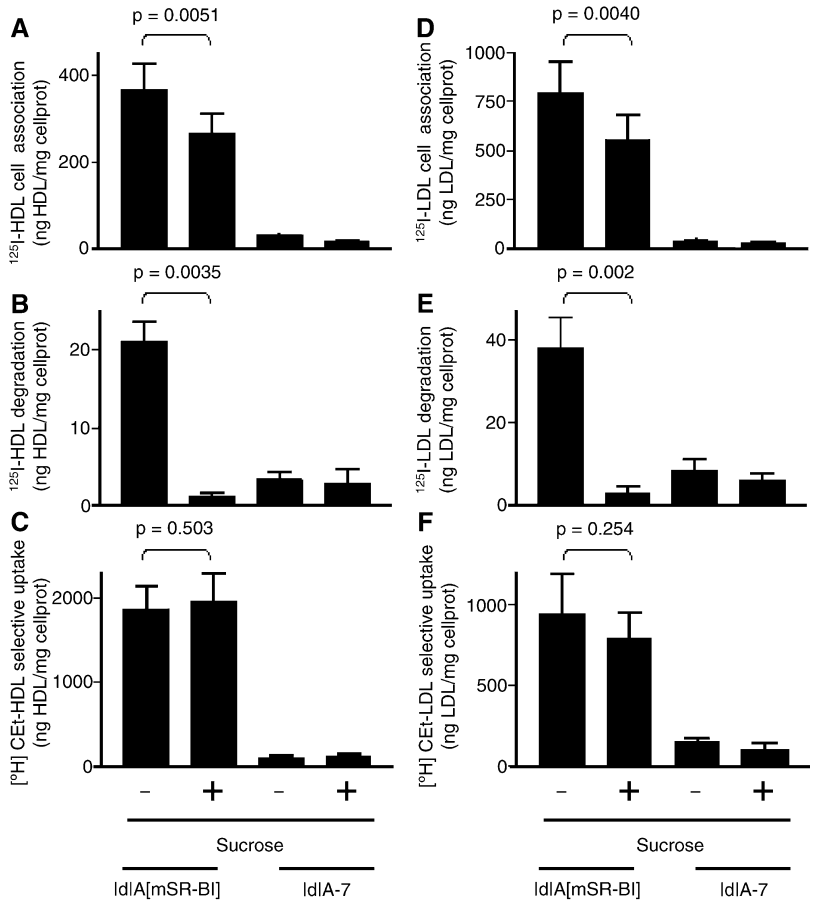

Fig. 3. Effects of endocytic block by hypertonic sucrose on cell association and degradation of ^{125}I-HDL and ^{125}I-LDL and on selective uptake of [^{3}H]CEt from [^{3}H]CEt-HDL or [^{3}H]CEt-LDL. Cells (ldlA[mSR-BI] or ldlA-7) were incubated for 30 min with the control medium (−) or hypertonic sucrose medium (+) followed by the addition for 60 min of ^{125}I-HDL, ^{125}I-LDL, [^{3}H]CEt-HDL, or [^{3}H]CEt-LDL (10 μg protein/ml final) in the presence or absence of a 40-fold excess of the respective unlabeled lipoproteins. The cells were washed and lysed to determine the total amounts of ^{125}I-HDL (A) or ^{125}I-LDL (D) associated with the cells (this value represents lipoprotein both bound at the cell surface and internalized). Media were harvested and used to determine the extent of ^{125}I-HDL (B) or ^{125}I-LDL (E) degradation. The selective transfer of [^{3}H]CEt from the [^{3}H]CEt-HDL (C) or [^{3}H]CEt-LDL (F) to the cells was calculated by subtracting the contributing amounts of [^{3}H]CEt contained in the lipoproteins associated with the cells. The mean values and standard errors determined in 6 independent experiments are shown. *P* values are shown for ldlA[mSR-BI] samples only.

more, the incubation of ldlA[mSR-BI] cells with ^{125}I-LDL results in a low level of lipoprotein degradation not observed in the untransfected control ldlA-7 cells (Fig. 3E). Thus, mSR-BI mediates a low level of endocytosis and degradation of LDL.

As was the case for HDL, hyperosmotic treatment of ldlA[mSR-BI] cells also inhibited the mSR-BI-mediated internalization (Fig. 4G) and degradation of LDL (Fig. 3E) but did not interfere with mSR-BI-dependent selective uptake of [^{3}H]CEt from [^{3}H]CEt-LDL (Fig. 3F) or of DiD from DiD-LDL (Fig. 5). As expected, ldlA-7 cells did not support selective uptake from [^{3}H]Cet-LDL (Fig. 3F) or DiD-LDL (not shown), nor cell association and degradation of ^{125}I-LDL (Fig. 3D, E). Based on these observations, we suggest that the mSR-BI-mediated selective lipid uptake from LDL also does not require lipoprotein endocytosis.

4. Discussion

In vitro and in vivo studies have established that the HDL receptor SR-BI plays a key role in mediating the physiologically relevant cellular selective uptake of lipid from lipoproteins, and thus controls the metabolism of HDL [1]. The detailed molecular mechanisms underlying selective lipid uptake mediated by mSR-BI remain to be established. One area of controversy regarding the mechanism of mSR-BI-mediated selective lipid uptake has been the role of receptor-ligand endocytosis. In the current study we have shown that the inhibition of cellular lipoprotein endocytosis by either hypertonic sucrose treatment or K^{+} depletion does not prevent the mSR-BI-mediated selective uptake of lipids (cholesterol ethers or the lipophilic dye DiD) from either HDL or LDL. These results support our previous conclusion

that mSR-BI-mediated HDL binding and selective lipid uptake are intrinsic properties of the receptor that do not require the intervention of other proteins or traffic to specific intracellular compartments [15]. Furthermore, we found that the specific inhibition of mSR-BI-dependent lipid transport by the small molecules BLT-1 and BLT-4 does not involve the inhibition of HDL particle internalization mediated by mSR-BI. Our results are consistent with previous electron microscopic morphological studies that support the model of HDL particle internalization independent SR-BI-mediated lipid transport [22,23].

During the preparation of this manuscript, two reports appeared supporting the proposal that SR-BI-mediated lipid transport is independent of lipoprotein internalization. In the first study, Maxfield and co-workers [24] reported that the intracellular depletion of ATP in HepG2 cells increased the efficiency of selective lipid uptake mediated by human SR-BI from the HDL of the fluorescent unesterified cholesterol analogue dehydroergesterol, even though the endocytic uptake of HDL particles was blocked. They noted, however, that the dehydroergesterol intracellular trafficking pathway involved a rapid non-vesicular mechanism that might differ from that of the selective uptake of HDL's cholesteryl esters (or cholesteryl ether analogues). In the second study, Eckhardt et al. [41] compared the selective uptake and endocytic activities of mSR-BI and a naturally occurring murine splice variant called SR-BII in non-polarized transfected cells. SR-BII and SR-BI differ only in their short 45–47 amino acid C-terminal cytoplasmic domains [42]. The steady state ratios of the levels of intracellular-to-cell surface receptor and

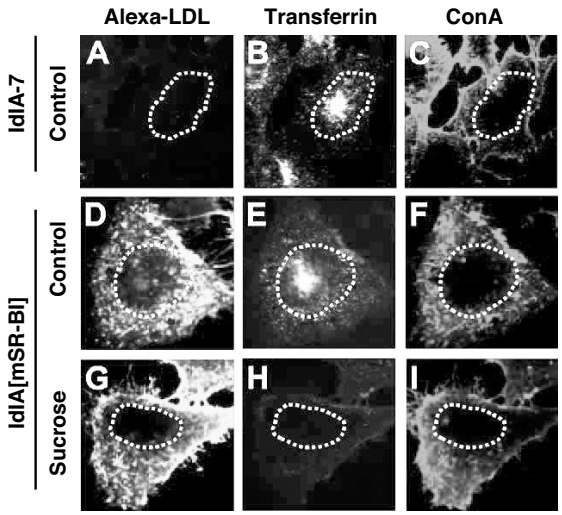

Fig. 4. Effect of hypertonic sucrose treatment on mSR-BI-mediated endocytosis of LDL. ldlA-7 and ldlA[mSR-BI] cells were treated with control medium (A–C) or medium containing hyperosmotic sucrose (D–F) as described in Fig. 1, but using Alexa568-labeled LDL (10 μg protein/ml final). Representative confocal sections are shown. The white lines circumscribe the cell interior.

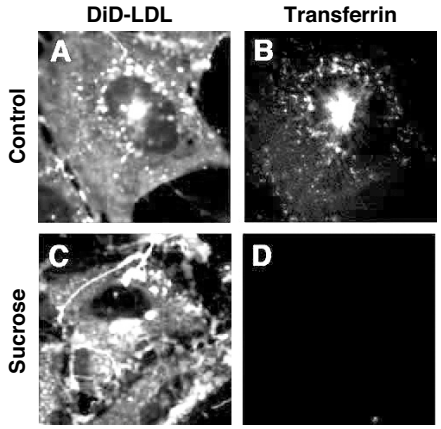

Fig. 5. Effect of hypertonic sucrose treatment on mSR-BI-mediated selective uptake of DiD from DiD-LDL. ldlA[mSR-BI] cells were treated with control medium (A, B) or medium containing hyperosmotic sucrose (C, D) as described in Fig. 1, but using DiD-labeled LDL (80 μg protein/ml final) (DiD-LDL). During the last 20 min of the incubation, Alexa488-transferrin was added. Cells were then washed and analyzed without fixation by three-dimensional fluorescence confocal microscopy. Representative confocal sections are shown.

associated HDL were substantially higher in the SR-BII expressing cells, possibly due to SR-BII's more rapid endocytosis (and/or slower recycling to the cell surface). Selective uptake by these receptors positively correlated with their levels of surface expression, but not with the levels of internalized HDL. Eckhardt et al. suggested that these data support the model that most of the selective uptake mediated by SR-BI occurred at the cell surface, although their study did not rule out the possible requirement for a rapid cycle of endocytosis and recycling that would result in a very small internal pool of SR-BI and its ligand that is able to efficiently support selective uptake. It remains possible that in some specialized cells (e.g. mammary epithelial cells [43]) mSR-BI-mediated endocytosis plays a role in lipid metabolism; however, in many, if not all cases, it clearly is not required for efficient selective uptake.

These reports, together with our study, demonstrate that the endocytosis of HDL or LDL is not required for mSR-BI-mediated selective uptake from these lipoproteins. Furthermore, our data strongly suggests that selective uptake occurs at the surface of the cell.

Acknowledgements

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CHAPTER V

Chemical genetic screening identifies sulfonamides that raise organellar pH and interfere with membrane traffic

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Chemical Genetic Screening Identifies Sulfonamides That Raise Organellar pH and Interfere with Membrane Traffic

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Chemical genetics seeks to identify small molecules that afford functional dissection of cell biological pathways. Previous screens for small molecule inhibitors of exocytic membrane traffic yielded the identification and characterization of several compounds that block traffic from the Golgi to the cell surface as well as transport from the endoplasmic reticulum to the Golgi network [Feng et al. *Proc Natl Acad Sci USA* 2003;100:6469–6474; Yarrow et al. *Comb Chem High Throughput Screen* 2003;6:279–286; Feng et al. EMBO Reports 2004: in press]. Here, we screened these inhibitors for potential effects on endocytic membrane traffic. Two structurally related sulfonamides were found to be potent and reversible inhibitors of transferrin-mediated iron uptake. These inhibitors do not block endoplasmic reticulum-to-Golgi transport, but do disrupt Golgi-to-cell surface traffic. The compounds are members of a novel class of sulfonamides that elevate endosomal and lysosomal pH, down-regulate cell surface receptors, and impair recycling of internalized transferrin receptors to the plasma membrane. *In vitro* experiments revealed that the sulfonamides directly inhibit adenosine triphosphate (ATP) hydrolysis by the V-ATPase and that they also possess a potent proton ionophore activity. While maintenance of organellar pH is known to be a critical factor in both endocytosis and exocytosis, the precise role of acidification, beyond the uncoupling of ligands from their receptors, remains largely unknown. Identification of this novel class of sulfonamide inhibitors provides new chemical tools to better understand the function of organelle pH in membrane traffic and the activity of V-ATPases in particular.

Key words: chemical genetics, membrane traffic, organelle pH, sulfonamides

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Chemical genetics seeks to identify novel small molecules that afford functional dissection of cell biological pathways (1–3). Such compounds are useful as bioactive molecular probes and allow further analysis of the relationship between target processes or proteins within cells and their cellular function. We have taken a chemical genetics approach to identify small cell-permeable molecules that block exocytosis (4,5). The exocytic or constitutive secretory pathway encompasses vesicular movement of newly synthesized membrane proteins and secretory components from the endoplasmic reticulum (ER) to the Golgi apparatus with final arrival at the cell surface (for resident plasma membrane proteins) or export (for secreted proteins). To identify small molecule inhibitors of this pathway, we employed a medium-throughput screen based on cell fluorescence imaging of a green fluorescent protein (GFP)-tagged vesticular stomatitis virus glycoprotein temperature-sensitive mutant (VSV-G^{ts045}) which transits this pathway in a well-defined and experimentally manipulable fashion (4,5). This investigation led to the discovery of Exo1(6B6) and Exo2(15E19), agents that block exit from the ER (4), as well as five other compounds (16D20, 30 N12, 4F17, 17G7, 16F19) that block exit from the Golgi (5). To further examine the selectivity of these compounds as inhibitors of specific steps of intracellular membrane traffic, we undertook the present study to explore their effects on the endocytic pathway.

The process of endocytosis brings extracellular material and plasma membrane proteins into cells from their surface through a series of membrane-bound compartments, often referred to as early, sorting and recycling endosomes. Internalized material is sorted and ultimately directed from these endocytic organelles for degradation in lysosomes or for return to the cell surface (recycling). Our knowledge of the endocytic recycling pathway is largely based on detailed analysis of the membrane traffic of

transferrin (Tf). This serum iron-binding protein associates with specific cell surface receptors to be internalized and delivered to the endocytic pathway. These binding interactions are facilitated at the extracellular pH of 7.4 with high ligand binding affinity (Kd $\sim$ 1–5 nM) (6,7). Tf–TfR complexes cluster into clathrin-coated pits to be delivered to early endosomes. The pH of endosomes is lowered by an ATP-dependent proton pump; under these conditions, the release of iron from Tf is facilitated through its interactions with Tf receptor (8). Unlike many other ligands, apoTf (devoid of its cargo iron) remains bound to its receptor and is therefore recycled back to the cell surface, where it can be released for further rounds of iron delivery (9–11). The vesicular movement of the Tf receptor through the early sorting and recycling endosomal pathway is well-defined (12).

In screening the small molecule inhibitors of exocytosis for their effects on cellular iron assimilation through the Tf cycle, we fortuitously identified a family of sulfonamide compounds that blocked uptake of iron via the Tf receptor pathway. Seven members of this family were characterized with IC_{50} values ranging from 0.1 μM to greater than 250 μM. Detailed study of one member, 16D10, revealed that treatment with this compound increases the pH of compartments of the endocytic pathway, thereby blocking both the release of iron from Tf and recycling of Tf receptors to the cell surface. Results from biochemical experiments presented here demonstrate that a potential target of the compounds is the V_1 domain of vacuolar ATPases (V-ATPases), which is responsible for pumping protons into the lumen of a variety of organelles, including endosomes, lysosomes and the Golgi apparatus. In addition, we found that members of the sulfonamide family can act as proton ionophores *in vitro*. While it is well-established that maintenance of organellar pH is a critical factor in both endocytic and exocytic membrane traffic, the precise role of acidification in these pathways remains largely unknown. The novel class of sulfonamide inhibitors therefore provides new chemical tools to better understand the function of organelle pH in membrane traffic and to study functional aspects of V-ATPases in particular.

Results

Compounds that block Golgi to plasma membrane traffic inhibit Tf-mediated iron uptake

In a recent high throughput screen of more than 10 000 chemicals from the DIVERSet E (ChemBridge Corp., San Diego, CA) for inhibitors of exocytosis, seven small molecules were identified that reduced the delivery of GFP-tagged VSV-G^{ts045} to the cell surface (4,5). Two compounds, 6B6 and 15E19, now called Exo1 and Exo2, respectively, were characterized to block exit from the ER (4). Five others, 16D20, 30 N12, 4F17, 17G7, and 16F19, did not affect VSV-G^{ts045} traffic from the ER to the Golgi apparatus, but instead inhibited the membrane protein's exit from the Golgi to the cell surface (5). Structures of these seven small molecule inhibitors are shown in Figure 1. When tested at 50 μM, these compounds reduced the amount of the viral glycoprotein delivered to the cell surface with inhibitory potencies that ranged between 95 and 30%, with 6B6 > 15E19 > 16D20–16F19–30 N12–4F17 > 17G7 (Figure 2).

To examine the effect of this set of inhibitors on the endocytic pathway, uptake of radioactive iron from Tf was measured in the presence of 50 μM of the compounds. After association with its receptor at the cell surface, Tf delivers iron to cells via the endocytic pathway. As shown in Figure 3, none of the drugs affected cell-associated ^{55}Fe at 4 °C (open bars), a temperature that blocks endocytosis of Tf. These data indicate that none of the drugs permeabilizes cells or in any way promotes non-endocytic uptake of iron from Tf. However, Tf-mediated uptake of ^{55}Fe at 37 °C (closed bars) was inhibited by 85% to 5% with an order of potency 16D20–30 N12–16F19 >> 17G7 > 4F17–15E19–6B6. This order of potency was completely different from inhibition of VSV-G traffic and confirms the selectivity of 6B6 (Exo1) and 15E19 (Exo2) to inhibit ER-to-Golgi transport. In contrast, inspection of the chemical features of two of the most potent inhibitors of both exocytic and endocytic traffic, 30 N12 and 16F19 (Figure 1), suggested a common structural basis for their effects on both pathways. Based on the presumption that these compounds might selectively target a cellular component functioning in both the endocytic and exocytic pathways, these compounds became a focus for further investigation.

Inhibition of Tf-mediated iron uptake by 30 N12 and 16F19 is dose-dependent and reversible

The dose–response of inhibition of Tf-mediated iron uptake by 30 N12 and 16F19 was determined. Both compounds effectively blocked iron uptake in a dose-dependent manner, with IC_{50} values of 0.1 and 2 μM, respectively (Figure 4). Moreover, inhibition by 30 N12 and 16F19 was reversible (Figure 5). In these experiments, cells were either first treated with vehicle solvent (DMSO) followed by a 1-h incubation with the inhibitor (open bars) or they were treated with the compounds for 1 h, followed by a 4-h recovery period with DMSO added (solid bars). Reversal of the inhibition of Tf-mediated iron uptake under the latter conditions also confirmed that there were no confounding solvent effects of vehicle treatment, and that these compounds were not toxic to cells.

Structurally related sulfonamides inhibit membrane traffic

Based on the chemical structures of 30 N12 and 16F19, five other sulfonamide compounds were selected from the ChemBridge DIVERSet E (Figure 6). The ability of members of this family of sulfonamides to inhibit uptake of ^{55}Fe

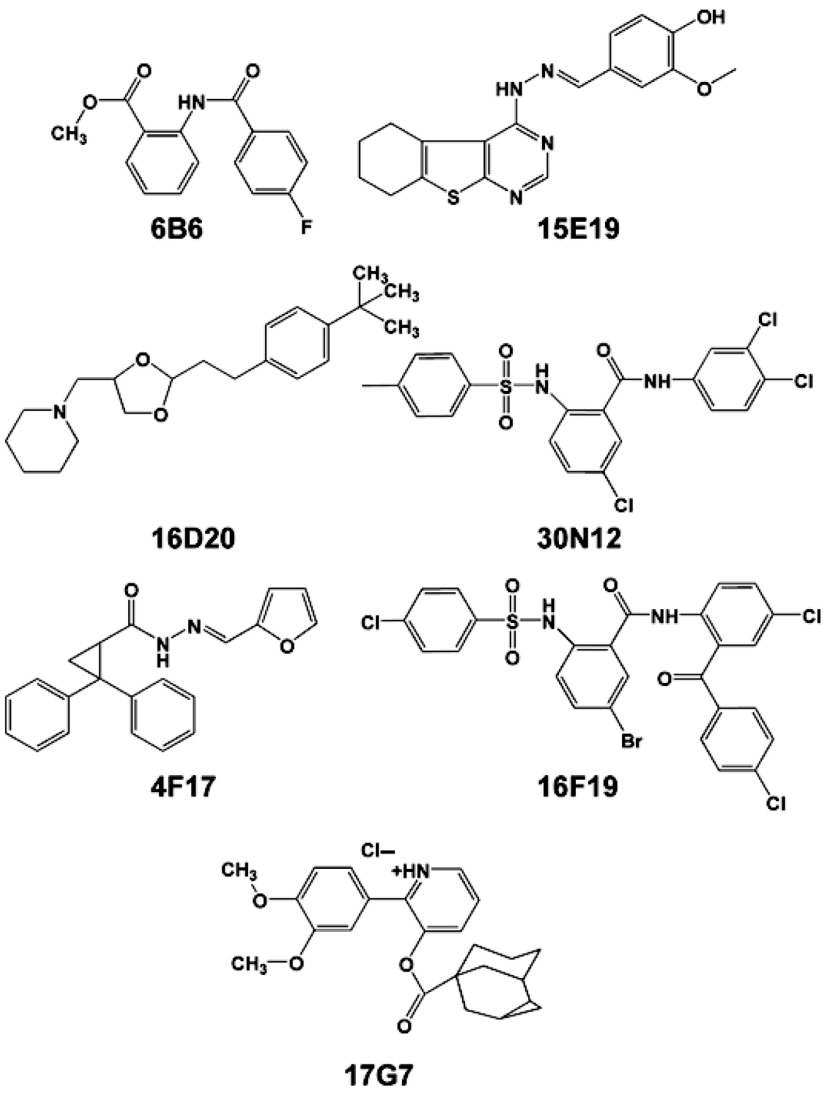

Figure 1: Chemical genetic screening identifies compounds that reduce exocytosis of GFP-tagged VSV-G^{ts045} to the cell surface. Structures of seven small molecule compounds were identified in a screen of $>10\,000$ chemicals from the ChemBridge DIVERSet E library to block secretion of VSV G. Screening conducted by Y.F. (4,5).

from Tf was determined in a series of dose–response experiments that established IC_{50} values from 0.1 μM (30N12) to $>$ 250 μM (23H9). Inhibition studies of the ability of these sulfonamide family members to block cell surface delivery of VSV G protein revealed a similar range of IC_{50} values (data not shown). From the relative potency of the compounds (30N12 $>$ 16L2–16F19–16D2 $>$ 16D10 $>>$ O-1 and 23H9), limited structure-activity information can be deduced. In more potent compounds, the sulfonamide is flanked by a single benzyl ring on the sulfonyl-group, whereas bulkier aryl groups are tolerated on the amide (e.g. 16F19). A converse configuration (23H9 vs. 16L2) significantly impairs inhibitory potency. Chloro- and/or nitroso substituents on the single benzyl ring off the sulfonyl-group do not appear to affect the compound's action (e.g. 30N12 vs. 16L2). On the aryl group adjacent to the amide, a halide is invariantly present in the para position; elimination of this halide greatly reduces the ability of the sulfonamide to inhibit both endocytosis and exocytosis (O-1). For more detailed functional studies, we focused on 16D10 since it has a structure representing the most significant aspects of this class of compounds: both chloro- and nitroso-groups are present on an aryl ring adjacent to the sulfonyl-group and a halide is para to the amide on a benzyl group, which itself is connected via an amide linkage to a second benzyl ring. As a control for some of these experiments, we employed O-1, which lacks the halide and nitroso constituents and displays an IC_{50} value for inhibition of ^{55}Fe uptake that is one order of magnitude greater than 16D10.

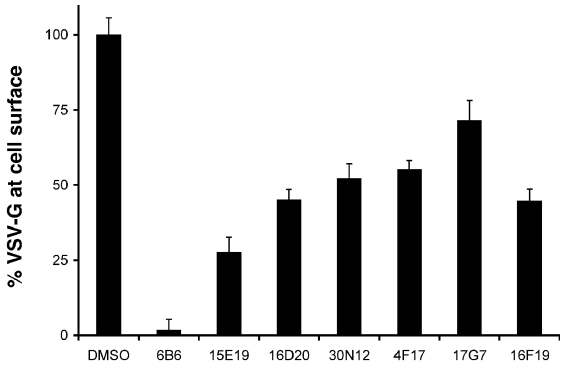

Figure 2: Inhibition of VSV-G^{ts045} exocytosis. To examine the effects of chemicals shown in Figure 1, BSC1 cells were transduced with adenovirus to express GFP-tagged VSV G^{ts045} and grown overnight at 40 °C. Prior to transfer to 32 °C, 50 μM of each compound was added to the media for 1 h. After 3 h incubation at 32 °C, the amount of VSV G^{ts045} at the cell surface was measured by incubating with monoclonal VSV-G antisera as detailed in the Materials and Methods section. As a control for nonspecific binding, cells were also treated with 10 μM brefeldin A, a drug known to completely block transport to the cell surface. After subtraction of background (measured in the presence of brefeldin A), data were normalized to the total fluorescence signal detected by GFP expression. Shown is the percentage of surface VSV-G relative to the total GFP signal ($n = 9$; ± SEM). Experiments performed by Y.F.

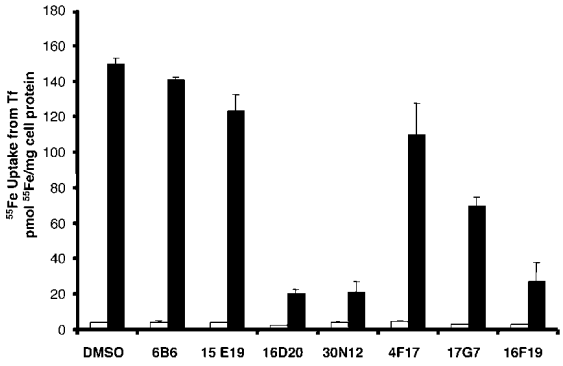

Figure 3: Inhibitors of the exocytic pathway block ^{55}Fe uptake from Tf. HeLa cells were treated with 50 μM of inhibitors shown in Figure 1 for 4 h at 37 °C (solid bars) or 4 °C (open bars) in the continued presence of 40 nM ^{55}FeTf. After washing, cells were lysed with 0.1% Triton X-100 containing 0.1% NaOH and cell-associated ^{55}Fe was determined by liquid scintillation counting. Results were normalized to protein concentrations to determine pmol ^{55}Fe/mg cell protein. The means of duplicate determinations are shown from a single experiment with similar results obtained on several occasions. Experiments were performed by J.X.B and P.D.B.

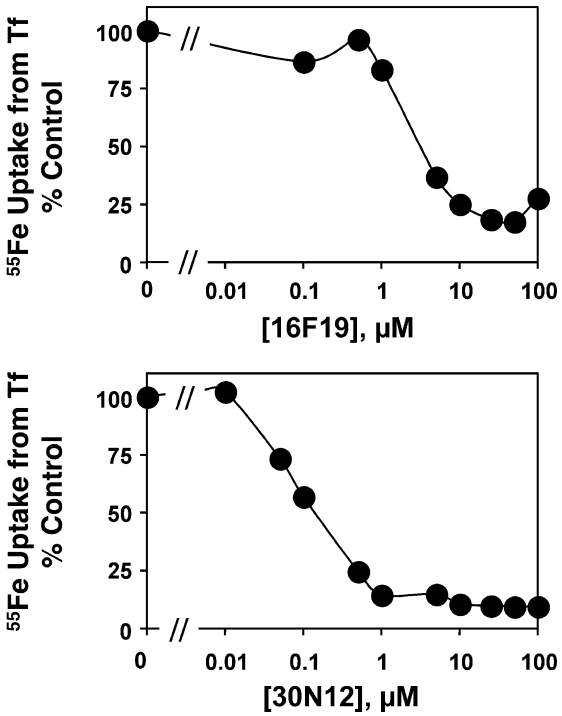

Figure 4: Dose–response to 16F19 and 30N12. Inhibition of ^{55}Fe uptake from Tf was determined exactly as described for Figure 1. HeLa cells were incubated with the concentrations of inhibitors and the amount of ^{55}Fe taken up by the cells was normalized to control cells which were treated with vehicle (DMSO) alone. Experiments performed by J.X.B and P.D.B.

16D10 does not inhibit non-Tf bound iron (NTBI) uptake or ER-to-Golgi traffic

To test whether the inhibition of iron assimilation was linked to the Tf–Tf receptor (TfR)-dependent pathway, the effect of 16D10 on NTBI uptake was examined. NTBI uptake involves transport of the metal directly across the cell surface in the absence of Tf, and does not depend on endocytosis (13). As shown in Figure 7, NTBI uptake was unaffected by the presence of 50 μM 16D10. From these data, we conclude that 16D10 does not act as a membrane perturbant or iron chelator to disrupt Tf-mediated delivery and cellular iron assimilation from this pathway. These results also indicate that inhibition of Tf-mediated iron uptake by the sulfonamide is unlikely to be caused by cellular ATP depletion, since NTBI uptake is an energy-requiring process (14). To further examine the selectivity of 16D10, we took advantage of GFP-tagged VSV-G^{ts045} to follow its movement by fluorescence microscopy. This temperature-sensitive mutant accumulates in the ER until cells are placed at permissive temperature (32 °C). Under these conditions, most of the GFP-tagged protein is observed in the Golgi apparatus after a 30-min incubation (upper panels, Figure 8). Treatment of cells with inactive O-1 or active 16D10 did not alter transport of the VSV G

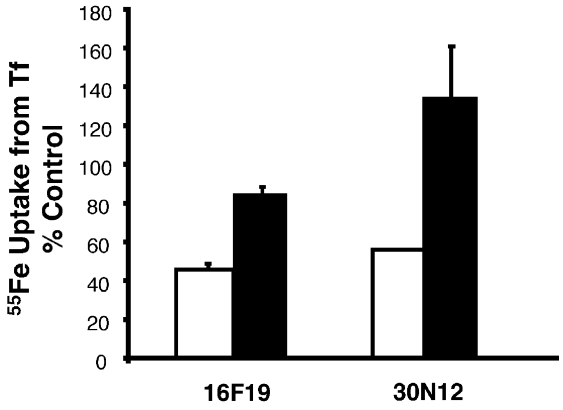

Figure 5: 16F19 and 30N12 inhibit ^{55}Fe uptake from Tf in a reversible manner. HeLa cells were either first incubated with 2 μM 16F19 or 0.5 μM 30N12 for 1 h followed by a 4-h recovery incubation with 0.5% DMSO after removal of the drug, or cells were first incubated for 4 h with 0.5% DMSO followed by a 1 h incubation with sulfonamides. Cells treated under both conditions were then incubated with 40 nM ^{55}Fe-Tf for 1 h. During the 1h assay, cells continued to be incubated either with DMSO (closed bars) to test reversibility (closed bars) or with sulfonamide to directly inhibitory potency in assay (open bars). ^{55}Fe uptake was normalized to protein content and expressed as percentage control (cells incubated with 0.5% DMSO in the absence of sulfonamide). Experiments performed by P.D.B.

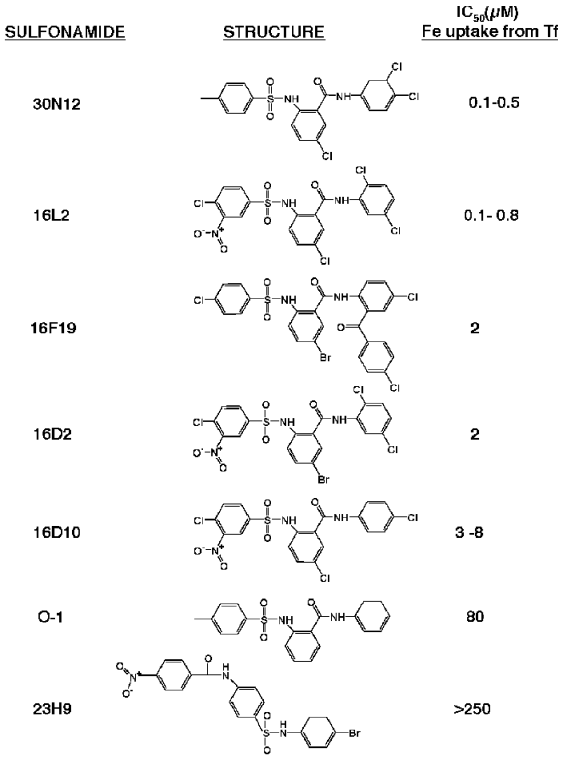

Figure 6: Family of sulfonamide inhibitors of membrane traffic. Based on the structures of 30N12 and 16D19, several additional compounds were screened for their ability to inhibit membrane traffic. Dose–response curves for inhibition of ^{55}Fe uptake from Tf were measured as described for Figure 4. In addition to 30N12 and 16F19 (5), structures of 16L2, 16D2, 16D10, 23H9 and O-1 are shown along with the relative IC$_{50}$ values determined for inhibition of Tf-mediated iron uptake. IC$_{50}$ values were determined as described in Materials and Methods using GraphPad PRISM3 software. Experiments performed by J.X.B and P.D.B. with data analysis by T.J.F.N.

protein to this compartment. Longer incubation times (2 h) resulted in the appearance of fluorescence at the cell surface; however, the presence of 16D10 significantly reduced traffic of the GFP-tagged protein to the plasma membrane (lower left panel, Figure 8). The observation that 16D10 does not block ER-to-Golgi transport is consistent with previous results obtained for 30N12 and 16F19 (5) and bolsters the idea that the sulfonamides selectively inhibit specific steps of membrane traffic involved in transport to the cell surface.

16D10 reduces Tf endocytosis due to receptor down-regulation

Endocytic uptake of Tf is initiated at the cell surface upon receptor binding, with internalization by clathrin-coated vesicles followed by delivery of the ligand to early or sorting endosomes. Control experiments confirmed that the sulfonamides did not interfere with Tf binding to its receptor (data not shown). To test whether subsequent steps of Tf endocytosis were affected, internalization of alexa594-labeled Tf was followed by fluorescence microscopy. The results in Figure 9 demonstrate that endocytic uptake of Tf continued in the presence of 10 μM 16D10 (left panel). Notably, there was no change in the morphology or distribution of endocytic compartments containing the fluorescent probe in cells treated with the compound. However, a significant decrease in the amount of ligand internalized was observed relative to control cells treated with vehicle (DMSO) or the inactive sulfonamide O-1. Thus, the pattern of alexa594-Tf traffic through the endocytic pathway is normal but the amount of ligand internalized is dramatically reduced by 16D10.

To evaluate whether reduced uptake is the result of a reduction in the number of Tf receptors on the plasma membrane, ^{125}I-Tf binding studies were performed (Figure 10, upper panel). Treatment with 10 μM 16D10 for 30 min induced a loss of $>60\%$ surface binding sites. Moreover, the number of surface low density lipoprotein (LDL) receptors was also reduced in cells treated with 16D10 (Figure 10, lower panel), indicating that the observed effects were not unique to the Tf receptor. Control experiments also confirmed that the latter observations did not result from altered LDL binding in the presence of 16D10 (data not

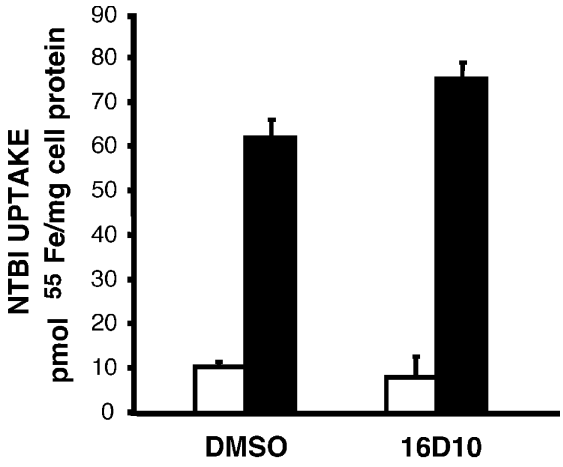

Figure 7: 16D10 sulfonamide does not inhibit NTBI uptake. The uptake of ^{55}Fe was measured in the absence of Tf by using a chemical chelate with NTA (1 : 4 ratio) to present the cation to HeLa cells. To measure nontransferrin bound iron (NTBI) uptake, cells were preincubated with 50 μM of the indicated compounds for 30 min prior to addition of 2 μM ^{55}FeNTA. After 1 h incubation at either 4 °C (open bars) or 37 °C (closed bars), uptake was quenched by placing the cells on ice and incubating with unlabeled 1 mM FeNTA to displace surface-bound iron; lysates were collected to measure cell-associated radioactivity and protein to calculate pmol ^{55}Fe/mg cell protein. Shown are the means of duplicate determinations from a single experiment with similar results obtained on several occasions. Experiments performed by J.X.B and P.D.B.

shown). From these combined results, we infer that the sulfonamide perturbs the endocytic recycling pathway to induce receptor down-regulation.

16D10 impairs Tf receptor recycling

A reduction in the amount of receptors on the cell surface can result from an accelerated rate of endocytosis, a reduced rate of exocytic return to the cell surface, or a combination of both perturbations. Experiments measuring the endocytic rate constant by the In/Sur method (15) indicated that Tf receptor internalization was not up-regulated by 16D10, as would be the case if the reduction in receptors on the cell surface was a consequence of altered internalization. However, recycling of Tf receptors back to the cell surface was significantly impaired in the presence of this compound. In these experiments, the release of internalized ^{125}I-Tf was monitored in the presence of 100 μM desferrioxamine (an iron chelator) and 3 μg/mL unlabeled Tf to prevent 'treadmilling' or recapture of ^{125}I-Tf back into the cells. The rate of ^{125}I-Tf release was greatly reduced in the presence of 16D10 (Figure 11, panel A) and from a series of experiments ($n = 4$), a near 10-fold reduction in the recycling rate constant was determined (Figure 11, panel B).

Sulfonamides alter endosomal and lysosomal pH

Previous studies have shown that flux of receptors and membrane components through the recycling pathway is slowed when the endosomal pH is raised (11,16–18). In addition, an intracellular retention or accumulation of recycling receptors is observed, an effect similar to the down-regulation of surface Tf and LDL receptors induced by 16D10 (Figure 10). We therefore tested whether the sulfonamides alkalinized Tf-containing compartments using a fluorescence ratio imaging method to determine the pH of recycling endosomes with doubly labeled Rh/Fl-Tf (17). 16D10 promoted an increase in Fl fluorescence, consistent with a rise in endosomal pH (Figure 12, panels A and B). Using methylamine to equilibrate cells with buffers of

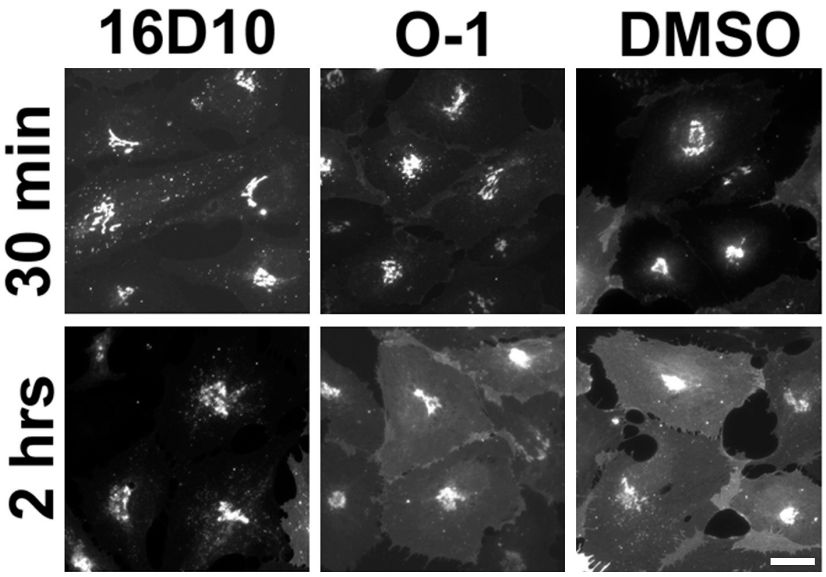

Figure 8: Sulfonamides block VSV-G^{ts045} transport from the Golgi apparatus to the plasma membrane. Cells were transduced to express VSV-G^{ts045} and incubated as described for Figure 2 except that 100 μM of 16D10 or O-1 were added before transfer to 32 °C. After 30-min and 2-h incubation periods, the cells were fixed with 3% formaldehyde and images of GFP-tagged VSV-G^{ts045} were collected using a 40× lens. Scale bar = 10 μm. Experiments performed by Y.F.

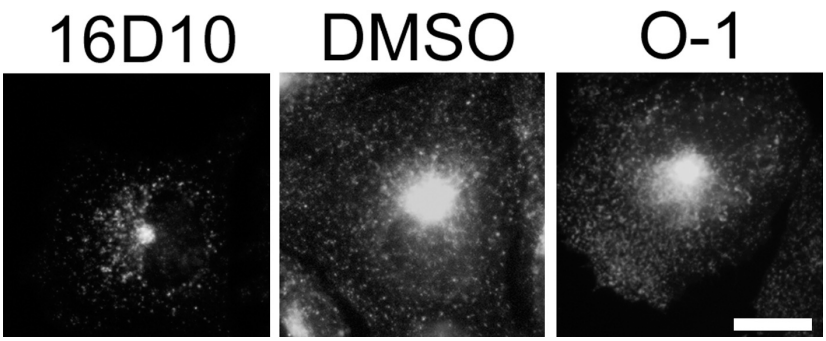

Figure 9: 16D10 sulfonamide reduces entry of Tf into cells. TRVb-1 cells were pretreated with 10 μM 16D10, O-1 or DMSO for 30 min and then 40 μg/mL Alexis594-labeled Tf was added for an additional 30 min. Cells were fixed with 3% formaldehyde and fluorescence images were collected using the same exposure times with a 63× oil immersion lens. Scale bar = 5 μm. Experiments performed by T.J.F.N.

known pH, a standard in-cell calibration curve was constructed (Figure 12, panel C). From the values of Rh/Fl ratios in control vs. 16D10-treated cells, it was determined that the pH of recycling endosomes was raised from 6.4 to 7.0 in the presence of the sulfonamide. These results are consistent with the model that receptor down-regulation and impaired Tf receptor recycling are induced by elevation of endosomal pH by the sulfonamide. Elevation of endosomal pH would also significantly impact the release of iron from Tf, since dissociation of the metal requires acidic conditions (8,10,11,16).

To study whether the sulfonamides could also alter lysosomal pH, the degradation of a fluid phase endocytic marker was followed. K562 cells were allowed to internalize exogenously added avidin-β-galactosidase at 20 °C to load the early endosomal compartment as previously described; a subsequent shift of temperature to 37 °C results in trafficking of this marker enzyme to the lysosomes where it is degraded (19). The amount of β-galactosidase remaining after the chase period can be measured in cell lysates by enzymatic hydrolysis of the fluorescent substrate 4-methylumbelliferyl-β-D-galactoside. Agents that are known to raise the lysosomal pH, such as NH_4Cl and the V-ATPase inhibitor bafilomycin A1, prevent the degradation of β-galactosidase (Figure 13). Similar to these lysosomotropic agents, addition of 10 μM 16D10 also inhibits the loss of β-galactosidase activity, consistent with the idea that the sulfonamides elevate the pH of the lysosomes.

Sulfonamides inhibit V-ATPase activity and act as potent proton ionophores

V-ATPases are required for the maintenance of the pH of different organelles, including the endosomal, lysosomal and Golgi compartments (20). Because 16D10 appeared to raise the pH of compartments in the endocytic pathway, the V-ATPase is an attractive candidate target for inhibition by the sulfonamides. To better understand the mechanism of action of these inhibitors, the effects of 16D2, 16D10 and O-1 on V-ATPase activity were studied biochemically (Table 1). The active sulfonamides, 16D2 and 16D10, inhibited hydrolysis of ATP by V_1-ATPase with IC_{50} values of 6 and 15 μM, respectively. These values correspond fairly well with their respective potencies for inhibition of iron uptake *in vivo* (Figure 6). Also consistent with the efficacy of sulfonamide inhibition *in vivo*, the relatively inactive compound O-1 inhibited only 30% of ATPase activity at a concentration of 250 μM. The IC_{50} values for inhibition of ATP hydrolysis were shifted by an order of magnitude, however, when the intact V_1V_0-ATPase was employed in these assays. This observation suggests that the assembly of the V_1 sector onto the V_0 domain may somehow partially protect the enzyme from sulfonamide inhibition, raising the possibility that the binding site for the sulfonamides may be in the stalk region at the V_1V_0 interface.

The effects of the sulfonamides on the ATP hydrolysis activity of the V-ATPase were unusual since most specific inhibitors, such as bafilomycin A_1, concanamycin A, and salicylihalamide A, do not inhibit ATP hydrolysis activity of dissociated V_1 or uncoupled V_1V_0 complex but instead inhibit ATP hydrolysis of V-ATPase by blocking the proton channel (21). To directly investigate the effects of the sulfonamides on the proton transport function of V-ATPase, *in vitro* assays were performed using proteoliposomes as previously described (21). During these studies, 16D2 was found to collapse the proton gradient generated by ATP-driven proton transport by the V-ATPase at a very low concentration (10 nM) as shown in Figure 14A. Further investigation using a protein-free liposome system to measure membrane potential-driven proton conductance revealed that the two effective sulfonamide compounds tested (16D2 and 16D10) act as proton ionophores in the nM range, whereas O-1 is more than a 1000-fold less potent (Figure 14B). This activity was unexpected and the potency of the sulfonamides' effects was quite surprising since most commonly employed proton ionophores are effective at ~1 μM. For example, the activity of the proton ionophore 1799 (bis-(hexafluoroacetonyl)acetone) at 1 μM

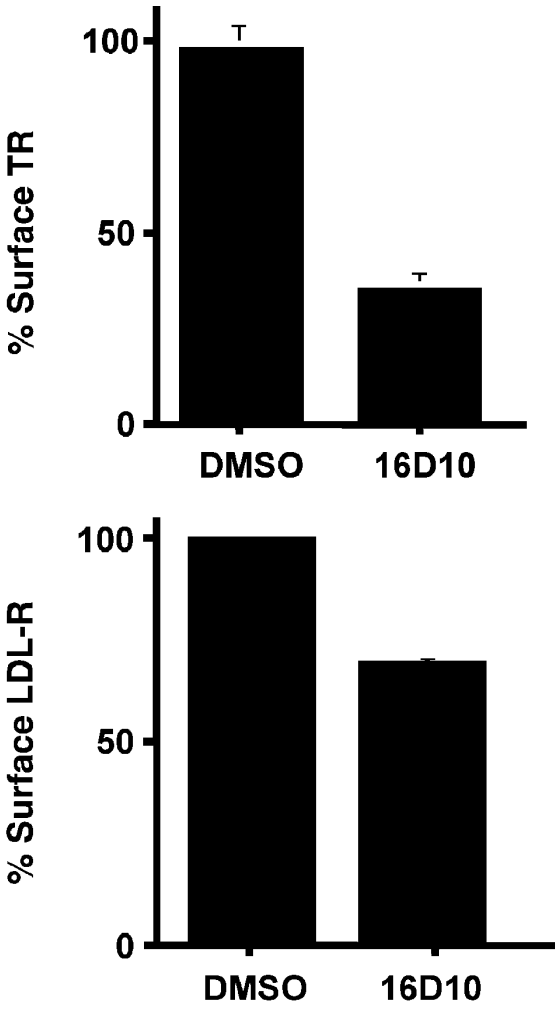

Figure 10: 16D10 sulfonamide promotes down-regulation of cell surface receptors. *Top panel:* TRVb-1 cells were preincubated in serum free media for 90 min at 37 °C, then treated with or without 10 μM 16D10 for 30 min at 37 °C. After cells were chilled on ice, surface ^{125}I-Tf binding was measured as described under Materials and Methods. Data shown are the average (± SD) of two experiments. The data are plotted as a fraction of the ^{125}I-Tf bound to the surface of control cells. *Bottom panel*: TVRb-1 cells were grown for 3 days in medium supplemented with lipoprotein deficient serum to up-regulate endogenous LDL receptor. On the day of assay, cells were washed twice in serum-free medium and pretreated with 10 μM 16D10 or DMSO alone for 30 min at 37 °C. Cells were then chilled on ice, and LDL labeled with ^{125}I on the lipoprotein moiety was added for 1 h on ice at a final concentration of 25 μg/mL. Non-bound LDL was washed away with phosphate-buffered saline and cells were lysed in 0.1 N NaOH for 30 min at room temperature. For each sample, an aliquot was counted by LSC and assayed for protein levels. Data are expressed as LDL receptor levels as a percentage of control. Note that saturating levels of ligands were used to measure cell surface binding for Tf and LDL receptors, 3 μg/mL and 25 μg/mL, respectively. Experiments performed by T.J.F.N and T.D.C.

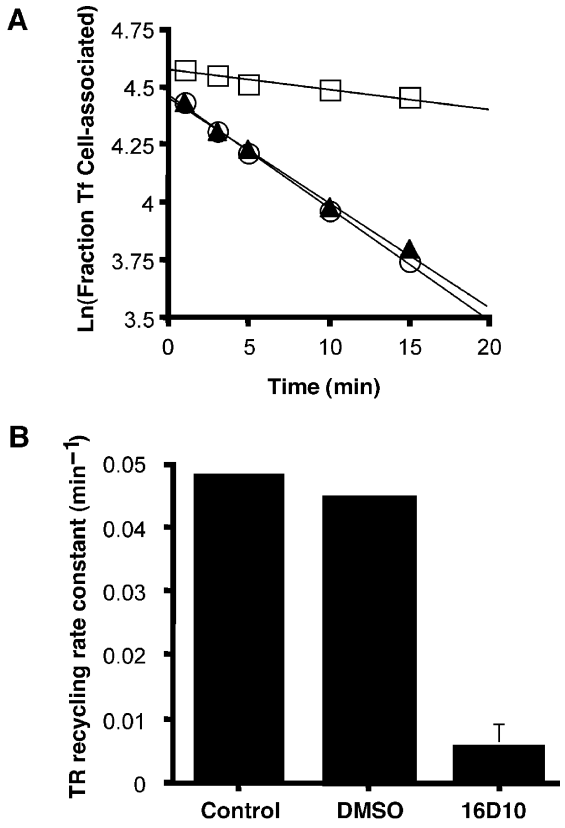

Figure 11: 16D10 sulfonamide impairs Tf receptor recycling. *Panel A:* TRVb-1 cells (treated with or without 16D10) were incubated with ^{125}I-Tf for 2 h at 37 °C to allow ligand internalization, then washed and incubated for the indicated time to allow recycling and release of Tf. The amount of ^{125}I-Tf remaining as a function of time is shown for control (open circles), cells treated with 0.5% DMSO (closed triangles) and cells treated with 10 μM 16D10 (open squares). *Panel B*: Recycling rate constant (min^{-1}) was determined from the slope of lines shown in the middle panel; shown is the mean value (± SD) determined in four separate experiments. Experiments performed by T.D.C.

is shown in Figure 14B. Because much higher concentrations of the sulfonamides are required to block intracellular membrane trafficking *in vivo*, exactly how these *in vitro* ionophore effects correlate with the influence of the compounds on cellular activities remains uncertain. Unfortunately, it is not possible to determine their precise influence on the V-ATPase proton pump function *in vitro* due to the confounding proton ionophore effects.

Discussion

Chemical genetics holds the promise of providing small molecules to study biological processes in greater detail. For example, recent screening efforts resulted in the

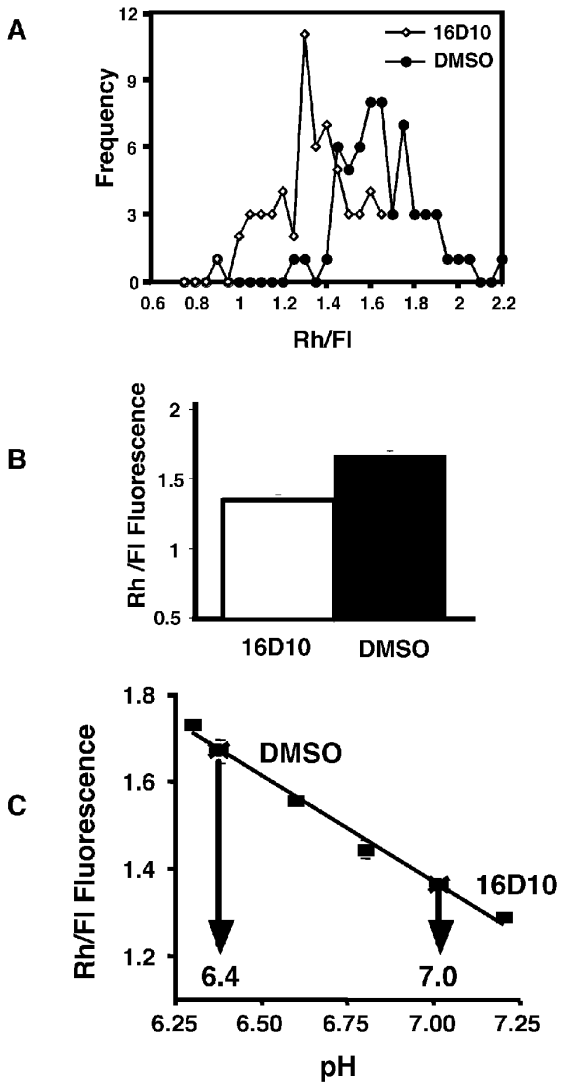

Figure 12: 16D10 sulfonamide alters endosomal pH. *Panel A*: Histogram of the distribution of Rh/Fl ratio of peri-centriolar recycling compartments of control and 16D10 treated cells. Compound 16D10 causes an increase in the Fl fluorescence, resulting in a shift of the distribution to the left. The data are from a representative experiment and 60 peri-centriolar recycling compartments from each condition were measured. *Panel B*: The average ± SEM Rh/Fl ratio of peri-centriolar recycling compartment from control and 16D10 treated cells ($n = 60$). *Panel C*: Estimation of endosomal pH based on the Rh/Fl ratio. A standard pH curve was constructed by determining the Rh/Fl ratio in cells whose endosomes were equilibrated to a known pH (squares). The Rh/Fl ratio measured in control and 16D10 cells can be converted to pH using this standard curve. The peri-centriolar endosomes in controls cells have a pH of ~6.4 and those in cells treated with compound 16D10 have a pH of ~7.0. Experiments performed by T.D.C.

discovery of two new inhibitors (Exo1 and Exo2) of ER-to-Golgi traffic (4,5). The effects of Exo1 appear similar to brefeldin A, a well-known fungal inhibitor of Golgi traffic (22). Unlike brefeldin A, however, its actions do not involve adenosine diphosphate (ADP)-ribosylation of Bars50 or inhibition of guanine nucleotide exchange activity of Golgi-associated ADP-ribosylation factors (ARFs) (4). Thus, Exo1 is a new chemical tool to help further dissect the mechanistic elements involved in ER-to-Golgi traffic. In the present study, we examined these and five additional compounds for potential effects on the endocytic pathway. From this analysis, two structurally related compounds, 30 N12 and 16F19, were discovered to block Tf-mediated iron delivery through the endocytic pathway. Both compounds were previously shown to interfere with Golgi-to-plasma membrane but not with ER-to-Golgi traffic (5), suggesting possible influences on membrane trafficking to the cell surface. 30N12 and 16F19 therefore became the founding members of a larger class of sulfonamide derivatives characterized in this investigation.

Members of the sulfonamide family inhibit endocytic uptake of iron via Tf–TfR complexes and the transport of VSV G protein from the Golgi to the plasma membrane in a dose-dependent and reversible manner. Structural relationships of this family include aryl groups linked across the sulfonamide, with a benzyl halide para- to the amide. While our structure–activity profiling was limited to a small set of seven compounds, searches of the ChemBridge DIVERSet E indicate that approximately 30 family members are available for study in this chemical library. Among the sulfonamides we have studied, IC_{50} values for inhibition of Tf-mediated iron uptake ranged from 0.1 μM to >250 μM. Similar values for inhibition of Golgi-to-plasma membrane traffic were observed, while none of the compounds appeared to block ER-to-Golgi transport.

The functional effects of one sulfonamide, 16D10, were studied in greater detail. The structure of 16D10 represents the most significant chemical attributes of the sulfonamide family. 16D10 inhibited Tf-mediated iron uptake with an IC_{50} ~3–8 μM but did not affect NTBI uptake. In cells treated with 16D10, endosomal pH was elevated from 6.4 to 7.0. Since release of iron from Tf requires endosomal acidification (8,10,11,16), dissociation of the metal would be blocked in the presence of this sulfonamide. Therefore, this effect accounts for the observed inhibition of Tf-mediated iron uptake and provides an explanation for why NTBI uptake is not affected by the compound. The alkalinization of endocytic compartments by 16D10 was also associated with a reduction in the number of surface Tf receptors due to impaired receptor recycling. This effect was not specific to the Tf receptor since down-regulation of the LDL receptor, another constitutively recycling receptor, was also observed. Previous studies of recycling endocytic traffic in the presence of pH-disrupting lysosomotropic agents have shown that Tf uptake is disrupted by alkalinization of intracellular organelles (7,9,23).

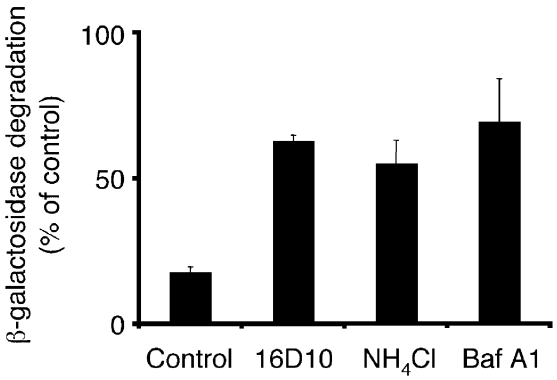

Figure 13: 16D10 prevents lysosomal degradation. K562 cells were incubated with β-galactosidase at 20 °C for 60 min to allow its internalization by fluid phase into endosomal (prelysosomal) compartments. After washing, cells were incubated for 30 min in the presence of 10 μM 16D10 or 1% DMSO vehicle control, 0.5 μM bafilomycin A1 (BafA1) or 20 mM NH_4Cl. The intracellular activity of internalized β-galactosidase following the chase period reflects access to lysosomes as is manifested by decreased β-galactosidase activity due to its proteolytic degradation within lysosomes. Results of duplicate samples were normalized to the total amount of activity before the chase period and are expressed as percentage control (DMSO vehicle treatment). Experiment performed by T.J.F.N.

Alterations in membrane traffic induced by weak bases like NH_4Cl, chloroquine or primaquine, include changes in the distribution of other recycling receptors as well. For example, monensin inhibits dissociation of ligand from the asialoglycoprotein receptor (24,25) such that lysosomal degradation is blocked (26). Weak bases also induce a loss of cell surface asialoglycoprotein receptors, with concomitant increases in intracellular pools (24–28). Similar to our observations that 16D10 promotes down-regulation of the LDL receptor, monensin also has been reported to block recycling of this receptor (29). The observation that 16D2 and 16D10 block the loss of endocytosed β-galactosidase activity due to membrane traffic is consistent with the idea that, very much like these other lysosomotropic agents, the influence on lumenal pH by these compounds is not specific to endosomes, since lysosomal protease activity also appears to be inhibited in cells treated with the compounds.

Table 1: Inhibition of ATPase activity of the catalytic sector V_1 and the intact V-ATPase by sulfonamide compounds. The ATP hydrolysis activity of the dissociated catalytic sector V_1 of bovine brain V-ATPase or the intact enzyme was measured in the presence of either $MgCl_2$ or $CaCl_2$, respectively. The IC_{50} of sulfonamides on the V-ATPase activity was obtained by titration of each compound. At the concentration of 250 μM, the highest concentration we have tested, the compound O-1 inhibited less than 50% of either ATPase activity. Experiments performed by J.W.

	IC_{50} (μM)	
Compounds	V_1 ATPase	Intact V-ATPase
16D2	6	27
16D10	15	32
O-1	> 250	> 250

Recent pharmacologic advances have provided specific inhibitors of the vacuolar H^+-ATPase to study the role of organelle pH in membrane traffic. Macrolide antibiotics bafilomycin A1 and concanamycins A and B have been identified to disrupt V-ATPase activity (30,31), although their precise mechanism of action is not yet entirely clear (20). A class of benzolactone enamides has also been identified to potently and selectively inhibit mammalian V-ATPase, but they are even less well-characterized (32). Of these V-ATPase inhibitors, bafilomycin A1 has been used most extensively in studies of membrane trafficking. Bafilomycin A1 inhibits the ATPase activity of the intact V-ATPase by blocking its proton channel (33) and subsequently raises the pH of both endosomal (17,18,34), and Golgi (35,36) compartments. Treatment with bafilomycin A1 markedly reduces the rate of TfR recycling and promotes receptor down-regulation (17,18,37,38). Similar to bafilomycin A1, 16D10 also raises endosomal pH to inhibit Tf-mediated iron uptake, induces receptor down-regulation, and impairs receptor recycling. It thus follows that the V-ATPase is a strong candidate target for inhibition by this compound. This model is also consistent with the fact that the sulfonamides do not block ER-to-Golgi transport but do inhibit Golgi-to-plasma membrane traffic of VSV-G protein (5). A pH gradient exists across the secretory pathway such that the *trans*-Golgi and the *trans*-Golgi network (TGN) are acidified (20). Similar to our observations of sulfonamide family members, bafilomycin A1 does not affect exit of VSV-G protein from the ER, but does cause its accumulation in the Golgi of virally infected cells (39). Prodigiosin-25, which uncouples V-ATPase driven proton transport, also inhibits cell surface transport of VSV-G (40).

Biochemical studies of isolated V-ATPase support the model that the sulfonamides may act by affecting its function. However, although the sulfonamides were found to inhibit hydrolysis of ATP by the V-ATPase *in vitro*, one surprise was that the sulfonamides also collapsed the proton gradient generated by ATP-driven proton transport of V-ATPase reconstituted in proteoliposomes. Studies using protein-free liposomes subsequently confirmed that the sulfonamides can act as proton ionophores. In fact, the proton ionophore activity of the compounds 16D2 and 16D10 *in vitro* is at least 100-fold higher than other ionophores commonly used in biochemical studies (e.g. 1799, FCCP, etc.). By comparing *in vitro* and *in vivo* data on the effects of the sulfonamides, however, it becomes apparent that the proton ionophore activity of these compounds must be 'neutralized' by cellular mechanism(s) *in vivo*. It seems likely that the 1000-fold concentration difference observed to elicit effects *in vivo* vs. *in vitro* reflects catabolism of the compounds in some manner that may

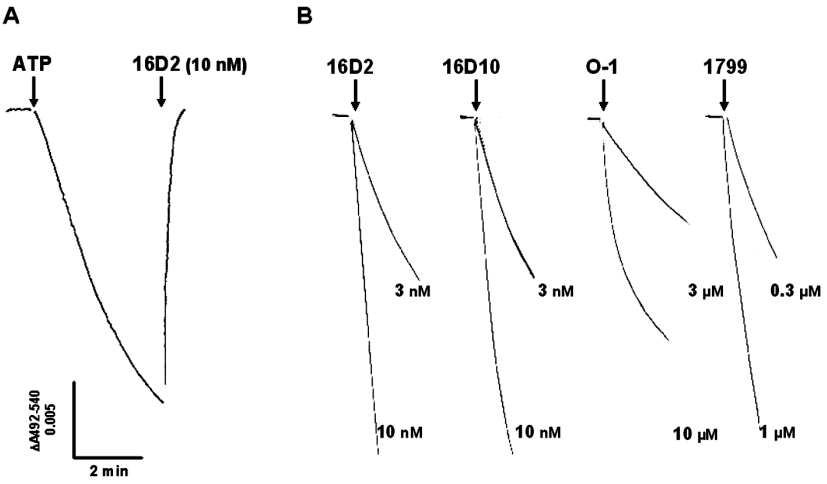

Figure 14: Proton ionophore activity of sulfonamides. *Panel A:* Bovine brain V-ATPase was reconstituted into proteoliposomes and assayed for ATP-driven proton transport activity. Sulfonamide analog 16D2 (10 nM) added at the end of the assay was found to collapse the proton gradient. *Panel B*: The proton ionophore activity of sulfonamides was measured as membrane potential-driven proton conductance in a protein-free liposome system. Sulfonamides and 1799 were added at the indicated concentrations. Experiments performed by J.W.

eliminate or significantly impair their ionophore activity. At this time, it remains uncertain whether these compounds act to raise the pH of intracellular compartments directly as proton ionophores or indirectly through inhibition of V-ATPase function. They could potentially act through both mechanisms, and it is also possible that their effectiveness as proton ionophores differs in different organelles such that this activity may be more responsible for some cellular effects than others. With respect to the inhibition of iron uptake which is thought to occur in endosomes, we note that the relative potencies of 16D2 and 16D10 for inhibition of ATP hydrolysis by V_1-ATPase *in vitro* are compatible with the relative IC_{50} values measured *in vivo*, while their proton ionophore activities at 1000-fold greater concentrations are almost identical *in vitro*. Thus, the model that the compounds inhibit ATP hydrolysis and thereby disrupt V-ATPase function is more compatible with the sum of our data for inhibition of Tf-mediated iron import.

Although we favor the view that the sulfonamides block V-ATPase function, it is curious that the IC_{50} values measured for inhibition of ATP hydrolysis by the intact V_1–V_0 complex are significantly higher than for the isolated V_1 sector alone. One possible explanation for these results arises from the inference that the compounds have a higher affinity to V_1 relative to the intact V-ATPase. The equilibrium between intact V-ATPase and its dissociated V_1 and V_0 domains in response to energy utilization and other cellular events has been well-established in yeast and insect systems (41,42), and is likely to be true in mammalian cells as well. In light of our data, the sulfonamides may inhibit the V-ATPase primarily by binding to free V_1, which could result from the disassembled inactive enzyme or which might even prevent V_1 from assembly with V_0. Although it can not be ruled out that the sulfonamides bind to the catalytic center of the transporter, wherein the pump's conformation changes when assembled with V_0, it is more likely that the binding site for these compounds is within the stalk region at the V_1–V_0 interface. In this regard, the sulfonamide compounds provide a particularly attractive tool to further study the structure, function, and regulation of V-ATPases.

Materials and Methods

Cell culture

BSC1 fibroblasts were cultured and transduced to express VSV G^{ts045} as previously described (4). For Tf uptake measurements, HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), and grown to ~ 60% confluency in 6-well plates prior to iron uptake studies. The CHO cell line, TRVb-1, was grown in 24-well plates with McCoy's medium containing 5% FBS to measure recycling and endosomal pH as described below. This cell line expresses the human Tf receptor but not the endogenous hamster Tf receptor, and has been extensively used for analysis of Tf receptor endocytic traffic (43). For ^{125}I-Tf surface binding and alexa-Tf uptake experiments, TVRb-1 cells were maintained in Ham's F12 medium with 10% FBS.

Small molecule inhibitors

Compounds were purchased from ChemBridge Corp. and dissolved in DMSO to a final concentration of 10 mM and stored at – 80 °C until use. During the course of this investigation, we discovered that the potency of the

compounds was greatly reduced if serum or bovine serum albumin was present in the assay medium, presumably because albumin may act as a scavenger to bind the lipophilic compounds, thus reducing their bioavailability.

VSV-G^{ts045} exocytosis

BSC1 cells transduced with adenovirus to express GFP-tagged VSV G^{ts045} were grown overnight at 40 °C in 96-well clear-bottom plates at a cell density of 10 000/well. Prior to transfer to 32 °C, compounds were added to the media at a final concentration of 50 μM and cells were incubated for 1 h at 40 °C. As a control, cells were also treated with 10 μM brefeldin A to completely block transport to the cell surface. After 3 h incubation at 32 °C, cells were fixed with 3% formaldehyde and stained with the monoclonal antibody 8G5 to detect GFP-tagged VSV-G^{ts045}-GFP at the cell surface using secondary goat antimouse labeled with alexa594. Cell-associated fluorescence was measured using an automated fluorescence microscope (Universal Imaging Corp., Downington, PA) with triple band-pass filters (excitation wavelengths of 360/490/570 nm; emission wavelengths of 460/530/625 nm). After subtraction of nonspecific binding of secondary antisera (measured in the presence of brefeldin A), the percentage of surface VSV-Gt^{s045} was calculated relative to the total amount of cell-associated GFP fluorescence. This surface/total ratio represents the extent in transport of GFP-tagged VSV-G^{ts045} to the cell surface.

Tf-mediated iron uptake

Human apoTf (Sigma Chemical Co., St. Louis, MO) was loaded with ^{55}Fe as previously described (13). HeLa cells were washed with serum-free media and incubated with 40 nM ^{55}Fe-Tf at 37 °C with or without inhibitors at the concentrations shown in the Figure legends. As a control, cells were incubated with vehicle alone (0.5% DMSO). At the end of the uptake period, cells were rapidly chilled on ice, washed with ice-cold phosphate-buffered saline (PBS) three times, then incubated with 40 μg/mL unlabeled Tf to displace surface-bound ^{55}Fe-Tf for 1 h at 4 °C. Cells were lysed with 0.1% Triton X-100 containing 0.1% NaOH. Cell-associated radioactivity was determined by liquid scintillation counting and cell protein was measured using the Bradford assay (44) to calculate pmol ^{55}Fe/mg cell protein.

Non-Tf-bound iron uptake

Iron uptake in the absence of Tf was measured using a chelate of ^{55}Fe with nitrilotriacetic acid (NTA) at a 1 : 4 ratio (13). HeLa cells were preincubated at 37 °C in serum-free media with 50 μM inhibitor for 30 min, then 2 μM ^{55}Fe-NTA was added for 1 h. At the end of the uptake assay, cells were chilled on ice, washed three times with phosphate-buffered saline, then incubated with 1 mM unlabelled Fe-NTA on ice to displace any surface-bound iron. Lysates were prepared and pmol ^{55}Fe/mg cell protein was determined as described above.

Alexa594-Tf uptake

TRVb-1 cells were first incubated at 37 °C for 2 h in serum-free Ham's F12 medium supplemented with 25 mM HEPES (pH 7.4) to remove any residual Tf bound to receptors. After preincubation with 10 μM 16D10 or vehicle (0.1% DMSO) in the same medium for 30 min at 37 °C, cells were incubated in the presence of 16D10 or DMSO for an additional 30 min with 40 μg/mL alexa594-Tf added to the media. After internalization of the fluorescent ligand, cells were washed in PBS containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$, fixed for 30 min on ice with 4% paraformaldehyde in PBS. Cells were analyzed for Tf uptake by fluorescence microscopy using a 63X oil immersion lens.

Down-regulation of cell surface Tf receptors

The effects of inhibitors on cell surface Tf receptors on the surface were determined by first incubating TRVb-1 cells in serum-free medium for 2 h at 37 °C in 5% CO_2. Compounds or DMSO were added during the final 30 min of this incubation. The cells were transferred to ice for 10 min to inhibit membrane trafficking, washed, and incubated in HEPES-buffered pH 7.2 balanced salt solution (HBSS) containing 3 μg/mL ^{125}I-transferrin to measure surface binding sites. All data were corrected for nonspecific ^{125}I-Tf binding, which was measured by incubating cells under the same conditions except that 600 μg/mL unlabeled Tf was added. After a 2-h incubation on ice, the cells were washed six times, solubilized and surface-bound radioactivity was measured by gamma counting.

Down-regulation of cell surface LDL receptors

TVRb-1 cells were grown in 24-well plates for 3 days in Ham's F12 medium containing 10% new born calf lipoprotein-deficient serum (a kind gift of Dr. Monty Krieger, MIT) to up-regulate expression of endogenous LDL receptors. On the day of the assay, cells (~40% confluency) were washed twice in serum-free Ham's F12 medium supplemented with 25 mM HEPES, pH 7.4, and pretreated with 10 μM 16D10 or DMSO (0.1%) for 30 min at 37 °C. The cells were then placed on ice, washed twice to remove compound, and then incubated for 60 min with 25 μg/mL of human LDL labeled with ^{125}I on the apoliprotein moiety (also generously provided by Dr. Krieger). Unbound ^{125}I-LDL was washed away with PBS and cells were lysed in 0.1 N NaOH. Cell-associated radioactivity was determined by liquid scintillation counting and cell protein was measured using the Bradford assay (44) to calculate the amount of LDL bound to the cells (ng ^{125}I-LDL/mg cell protein). Data was corrected for nonspecific binding determined in the presence of 40- fold excess nonlabeled LDL.

Tf receptor recycling kinetics

The kinetics of Tf receptor recycling in TRVb-1 cells were measured as previously described (45). Briefly, on the second day after plating into 24-well plates, cells were incubated with 3 μg/mL ^{125}I-Tf in serum-free McCoy's medium for 2 h at 37 °C in 5% CO_2. This medium contains

20 mM HEPES and 26 mM sodium bicarbonate. Compounds were added at 50 μM final concentration during the last 30 min of incubation. In control samples, DMSO (vehicle) was added to final concentration of 0.5%. At the end of the incubation period, the cells were washed once with serum-free McCoy's medium, washed with a mild acid wash at pH 5 for 2 min, and then washed three times with HBSS. The cells were then incubated in serum-free McCoy's media supplemented with 100 μM desferrioxamine and 3 μg/mL of unlabeled Tf. The release of Tf from cells was monitored as a function of time by collecting the medium and solubilizing cells to measure ^{125}I-Tf released and remaining in cells, respectively. The recycling rate constant for the Tf receptor was determined from plots of the natural log of the fraction of cell-associated Tf vs. time. Nonspecific binding was measured by incubating cells with 3 μg/mL of ^{125}I-Tf and 600 μg/mL unlabeled Tf. All data were corrected for nonspecific ^{125}I-Tf binding.

Endosomal pH measurements

TRVb-1 cells were plated in McCoy's 5A medium supplemented with 5% FBS on coverslip bottom dishes 2 days before experiments. On the day of the experiment, the cells were incubated with 20 μg/mL rhodamine/fluorescein-labeled Tf (Rh/Fl-Tf) for 90 min at 37 °C. Compounds or vehicle (0.5% DMSO) were added at 50 μM during the final 30 min of incubation. One dish was incubated for 90 min in serum-free McCoy's 5A medium without any additions to determine background fluorescence. At the end of the incubation, samples were washed four times over 1 min with HBSS, transferred to the microscope stage and imaged live. A field of cells was chosen in the rhodamine channel, and a rhodamine and fluorescein images were collected. Images from five fields for each condition were collected. Samples were examined sequentially and loading times were staggered to insure that the incubation periods were constant among the different samples.

To correlate the Rh/Fl ratio with pH values, an in-cell calibration curve was constructed. Cells were incubated with 20 μg/mL Rh/Fl-Tf for 90 min, washed four times with HBSS, and fixed for 5 min in 3.7% formaldehyde. Each sample was transferred to a different pH buffer (6.2, 6.6, 6.8, or 7.2) containing 40 mM methylamine. After a 1-h incubation, during which time the endosome pH equilibrates with the buffer pH, fluorescein and rhodamine images were collected from each dish (three fields per dish).

Images were collected on an Axiovert 200M inverted microscope using a 40×, 1.3 NA oil immersion objective (Carl Zeiss, Inc., Thornwood, NY), and a cooled CCD-camera 1300-V/HS from Princeton Instruments, Inc. (Monmouth Junction, NJ). In all experiments the exposure times were set such that less than 5% of the pixels were saturating in the brightest samples. Images were processed using Metamorph image processing software (Universal Imaging Corp.) as described previously (46). The intensity of the pixels of the endosomes was measured by encircling the peri-centriolar endosome recycling compartment. The average pixel intensity was determined and background corrected by subtracting average pixel intensity from dishes that were not incubated with Rh/Fl-Tf. The ratio of rhodamine to fluorescein was calculated per endosomal recycling compartment and averaged values are presented.

Measurement of endocytosed β-galactosidase activity

K562 cells were washed three times in PBS and suspended in uptake buffer (150 mM NaCl, 25 mM HEPES, pH 7.4, 1 mg/mL dextrose, 1 mg/mL bovine serum albumin [BSA]) with 0.5 mg/mL avidin-linked β-galactosidase as previously described (19). Internalization of the fluid phase endocytic marker was allowed to proceed for 60 min at 20 °C to permit accumulation into early endosomes. After extensive washing to remove any noninternalized marker, cells (5×10^6/mL) were resuspended in uptake buffer without BSA but with vehicle (1% DMSO) or 10 μM 16D10. For comparison, cells were also treated with 20 mM NH_4Cl or 500 nM bafilomycin A_1. After continued incubation at 20 °C for 30 min, control cells were chilled on ice, while a paired sample was warmed to 37 °C to permit traffic to later compartments of the endocytic pathway. After 60 min incubation, the latter samples were also placed on ice, and along with control samples, cells were washed three times in ice-cold PBS, resuspended in hypotonic breaking buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 85 mM sucrose, 20 mM EGTA), and snap-frozen in liquid N_2. Samples were stored at −80 °C until β-galactosidase activity was measured.

To obtain cell lysates, samples were thawed at room temperature, then placed on ice and vigorously vortexed for 1–2 min, then snap-frozen again. This procedure was repeated two times until > 90% of the cells were broken. Post-nuclear supernatants were collected upon centrifugation at 800 × *g* for 5 min at 4 °C. Aliquots of 10 μL of the cell lysates were removed for β-galactosidase assays. Lysates were solubilized with 1% octyl glucoside and enzyme assays were performed as previously described using the fluorogenic substrate 4-methylumbelliferyl-β-D-glactoside (19). Fluorescence (355 nm excitation; 460 nm emission) was measured using a SpectraMAX Gemini XS plate reader (Molecular Devices, Sunnyvale, CA).

Measurement of ATPase activity

ATPase activity was measured as the liberation of $^{32}P_i$ from (γ-^{32}P)ATP (Amersham) (47). In brief, purified V-ATPase from bovine brain (21), 1 μg protein for each assay, was first mixed with 2 μg phosphatidylserine and incubated at room temperature for 5 min. The ATP hydrolysis assay was started by addition of 200 μL assay solution (30 mM KCl, 50 mM Tris-MES, pH 7.0, 3 mM $MgCl_2$, and

3 mM [γ-^{32}P]ATP (400 cpm/nmol)) in the presence or absence of sulfonamide compounds at designated concentrations and continued for 15 min at 37 °C. For ATPase assay of the dissociated catalytic sector V_1 there is no need to incubate the enzyme with phosphatidylserine and Ca-ATPase activity was measured instead of Mg-ATPase activity, by replacing $MgCl_2$ with 3 mM $CaCl_2$. The ATP hydrolysis reaction was terminated by adding 1.0 mL of 1.25 N perchloric acid, and the released $^{32}P_i$ was extracted and counted in a Beckman scintillation counter as described (33).

Reconstitution of V-pump into proteoliposomes and measurement of proton translocation

The purified bovine brain V-ATPase was reconstituted into liposomes, which contain phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol at a weight ratio of 40 : 26.5 : 7.5 : 26, by the cholate dilution, freeze-thaw method (21). In brief, liposomes (200 μg) were added to 1 μg of V-ATPase and were well mixed. Glycerol, Na-cholate, KCl, and $MgCl_2$ were added to the protein–lipid mixture at final concentrations of 10% (v/v), 1%, 0.15 M and 2.5 mM, respectively. The reconstitution mixture was incubated at room temperature for 1 h, frozen in liquid N_2 for 1 min, thawed at room temperature and ready for assay. Proton translocation was measured using the Acridine orange quenching (48), which was conducted in an SLM-Aminco DW2C dual wavelength spectrophotometer and the activity was registered as $\Delta A_{492\text{-}540}$. The mixture was diluted into 1.5 mL of proton pumping assay buffer (150 mM KCl, 10 mM Na-tricine, pH 7.0, 3 mM $MgCl_2$, and 6.7 μM Acridine orange) in a spectrophotometer cuvette, which allows the formation of sealed proteoliposomes. The reaction was initiated by addition of 1.3 mM ATP (pH 7.0) and 1 μg/mL valinomycin and continued for 5 min. Inhibitors were added into the assay solution either prior to the start or at the end of the assay.

Measurement of proton ionophore activity

Proton ionophore activity of sulfonamides was examined by their ability to collapse the proton gradient generated by the proteoliposomes of V-ATPase as described above, and by a direct measurement of the membrane potential-driven proton conductance of these compounds in a protein-free liposomes system. The membrane potential was established by loading the liposomes with 150 mM KCl and diluting into a solution containing NaCl instead of KCl, which will generate a membrane potential, internal negative, in the presence of valinomycin, a potassium ionophore. This membrane potential will drive a proton influx if a mechanism for proton conductance is present, either a proton channel or a proton ionophore, which has been previously used to study the proton channel activity of V_0 (49). In brief, the liposomes were reconstituted as described above, except that a buffer without protein was used instead of V-ATPase. The liposomes were sealed by dilution into 150 mM KCl, 10 mM Na-tricine, pH 7.0, and 2 mM $MgCl_2$, followed by centrifugation at 100 000 × *g* for 1 h to precipitate the sealed liposomes that were loaded with KCl and suspended in a small volume of the same buffer. The proton conductance assay was performed using Acridine orange quenching as described above, except that the assay solution contains 150 mM NaCl in place of KCl. Specifically, an aliquot of the above sealed liposomes containing 100 μg lipids was added into an assay solution containing 150 mM NaCl, 10 mM Na-tricine, pH 7.0, 2 mM $MgCl_2$, 6.7 μM Acridine orange, and 1 μM valinomycin, and the compound to be assayed was added into the cuvette, after a stable baseline was established, to measure the proton conductance of the compound.

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CHAPTER VI

Discussion

The use of chemical genetics to study cellular transport

The research described in thesis has greatly benefited from the methodology of chemical genetics to probe the mechanisms of cellular lipid transport and of intracellular vesicular trafficking. The decision to use this approach finds its origin in the fact that small molecule inhibitors often act rapidly and reversibly. This makes them ideally suited to study complex dynamic processes in real-time in a manner that cannot be achieved by genetic methods.

Cellular lipid transport by SR-BI

Chapter II of this thesis describes the design and use of a high throughput small molecule screen to identify novel chemicals that modulate SR-BI mediated selective lipid uptake. For the purpose of the screen, HDL was loaded with fluorescent lipid probes to mimic the transfer of cholesteryl esters. Five compounds, two of which are structurally related, where found to inhibit selective uptake and they were named BLTs because they Block Lipid Transport. BLTs are specific for SR-BI and do not interfere with other aspects of membrane biology (endocytosis and exocytosis), with the integrity of the actin- or microtubule network or, with the exception of BLT-4, with cholesterol efflux of the unrelated lipid transporter ABCA1. The molecular target(s) of the BLTs is found within the cell, and it is not a component of the HDL particle.

Unexpectedly, BLTs did not block selective uptake by preventing the binding of HDL to SR-BI, by down-regulating the surface level of the receptor, or by preventing endocytosis of the lipoprotein. Instead, they increased the affinity of SR-BI for HDL, indicating that binding and lipid transport are tightly connected and not separate events. These results confirm the idea that HDL needs to form a productive complex with the receptor for selective uptake to occur (1, 2).

Under physiological conditions, depletion of lipids from HDL by selective uptake reduces the size of the lipoprotein. Because SR-BI has a lower affinity for small HDL, this permits the release of HDL, thereby liberating the receptor for another round of uptake from a new incoming lipoprotein (1, 3-6). One plausible explanation is that BLTs are inhibitors of the actual lipid transport step. Accordingly, and in agreement with this model, a consequence of a BLT imposed block in selective uptake is that HDL does not shrink, resulting in the observed increase in the affinity of SR-BI for HDL and a decrease in the off-rate of the lipoprotein. Alternative scenarios, including one in which compounds alter the binding of HDL to SR-BI so that selective uptake is unable to proceed, cannot be excluded at this moment.

SR-BI mediated cellular cholesterol efflux is less sensitive (higher EC_{50}) for BLT-3, -4 and -5 than the selective uptake pathway. These differences may reflect the chemical dissimilarities between core cholesteryl-esters and unesterified cholesterol found in the outer shell of HDL. This possibility was addressed by measuring SR-BI mediated uptake of radioactive unesterified cholesterol from HDL. These experiments indicate that cholesterol *selective uptake* from HDL is more potently blocked by these three BLTs than cellular cholesterol *efflux* (but cholesteryl-ether uptake is inhibited with similar efficacy) (not shown). Thus, the differences in EC_{50} of BLTs for selective uptake and efflux hint to differences between the uptake and efflux pathways that can be probed with chemical genetics. One possibility is that this subset of BLTs interferes differentially with the ability of an unknown protein (‘*protein A’)* to assemble into two different complexes, one involved in selective uptake and one that facilitates cholesterol efflux. Perhaps these BLTs disrupt more potently the ‘uptake complex’ and, hence, the selective uptake pathway. This may result from BLTs having a higher affinity for the ‘uptake complex’, or from an intrinsic lower stability of the complex that renders it more prone to collapse upon binding of the small molecules. A (controversial) candidate for this

complex is the assembly of the cholesterol chaperone caveolin, cyclophilin A and cyclophilin 40, which is claimed to facilitate selective uptake when it associates with Annexin II (7), and to be involved in efflux when it associates with HSP56 (8). A variation on this theme is that '*protein A'* switches its activity between selective uptake and efflux, depending on a particular post-translationally modification (e.g. phosphorylation, myristolation), and the compounds could interfere more potently with the 'uptake state' of the protein. Yet another possibility is that the BLTs bind with different affinities to two related but non-identical proteins involved in uptake and efflux respectively.

The role of endocytosis in SR-BI mediated selective uptake

It is unclear whether SR-BI mediated selective uptake occurs at the surface, in an intracellular compartment, or both. Blocking cellular internalization of HDL and LDL by generic inhibitors for endocytosis did not affect selective uptake, or the subsequent movement of (fluorescent) lipids into the interior of the cell (Chapter IV). Although it remains a formal possibility that uncoupling of endocytosis and selective uptake is cell-type dependent, these data suggest that selective uptake of cholesteryl esters *in vivo* likely occurs at the cell surface. This implies that previous reports of internalized HDL are not relevant to lipid transport, but merely reflects the low levels of (constitutive) endocytosis of SR-BI, perhaps moving to the lysosomal compartment at the end of its life cycle. This would be consistent with the very low amount of HDL that is degraded in an SR-BI dependent manner. Alternatively, the internalized SR-BI and HDL may recycle to the surface in a manner analogous to the transferrin receptor (9, 10).

SR-BII is an alternative splice form of SR-BI, but its physiological role is unknown to date. SR-BII and SR-BI express different intracellular C-terminal domains, which are 44- and 47 amino acids long respectively (11). This alternative tail allows SR-BII to efficiently internalize HDL into an intracellular environment that seems to support lipid transfer poorly (12). SR-BI, SR-BII and HDL are all claimed to enter the same intracellular compartment (9, 10, 12, 13), making it likely that intracellular selective uptake from HDL internalized by SR-BI is a similar inefficient process. Amino acid swap analysis between the C-terminal domains of SR-BI and SR-BII may assist in the elucidation of the underlying endocytic machinery.

Functional comparison between the lipid transporters SR-BI and ABCA1

Small molecules were used to probe for similarities in the mechanism of lipid transport of SR-BI and the structurally unrelated transporter ABCA1. In addition to its effect on SR-BI dependent transport, BLT-4 also abolished cholesterol efflux by ABCA1. The effect of BLT-4 was not restricted to a single member of the ABC family as it also inhibits NorA, an ABC transport protein in *Staphylococcus aureus* (14), suggesting that the compound targets a conserved feature in ABC proteins. Conversely, glyburide, a generic inhibitor of the ABC family (15-25) that blocks ABCA1 mediated cholesterol efflux (22-25), phenocopies the effect of BLTs on SR-BI. Glyburide prevented SR-BI mediated selective uptake and cholesterol efflux and increased the affinity of SR-BI for HDL. BLT-4 blocks SR-BI and ABCA1 mediated cholesterol efflux with similar potency and the same is true for glyburide (but BLT-4 is the more potent of the two drugs). This suggests that SR-BI and ABCA1 may share similarities in their mechanisms of lipid transport. Short of the possibility that both compounds directly target SR-BI and ABCA1, an intriguing possibility is that an ABC transporter protein of unknown identity ('ABC-X') is the rate-limiting step in SR-BI mediated lipid transfer. ABCA1 could not be detected in our SR-BI expressing cells, making it unlikely that ABCA1 participates in SR-BI activity. In

fact, it has been proposed that SR-BI and ABCA1 have competing roles, in which SR-BI promotes the reuptake of cholesterol effluxed to apoA-I by ABCA1 (26).

SR-BI reconstituted in liposomes can mediate selective uptake without the help of other proteins (27), suggesting that the putative ABC-X is not required for the lipid transfer step, but rather may modulate it. To date, forty-eight different human ABC proteins have been identified (28), and the number of ABC transporters that are involved in lipid transport or otherwise affect cholesterol homeostasis continues to grow. An RNAi-based approach to systematically knock-down different ABC proteins in SR-BI expressing cells could begin to provide answers.

How could this unknown ABC transporter modulate the activity of SR-BI? ABCA1 is proposed to create a specific lipid microenvironment to allow docking of apoA-I and subsequent cholesterol efflux (29, 30). If true, and assuming the hemi-fusion model of SR-BI activity is correct (2), it can be speculated that hemi-fusion of the outer shell of HDL with the surface membrane requires rearrangement of the cellular lipid bilayer by ABC-X in a manner similar to ABCA1, and this may be prevented by BLT-4. It is noteworthy that SR-BI was proposed to create a similar distinct lipid milieu, but recent genetic mutations in SR-BI have shown that this is not required for lipid exchange between SR-BI and lipoproteins (31). Thus, while the inhibitory mechanisms of BLTs-1, -2, -3 and -5 remain obscure, these results suggest that an ABC protein is the molecular target of BLT-4.

Physiological effect of BLTs

One of the outstanding questions concerns the impact of BLTs on SR-BI related physiology. Alterations in SR-BI expression in mice have profound effects on cellular cholesterol homeostasis and lipoprotein metabolism, and the absence of SR-BI increases the risk for cardiovascular disease and atherosclerosis (32). Indeed, SR-BI expressed in the liver and steroidogenic tissues is needed to remove cholesteryl-esters from HDL in the final phase of reverse cholesterol transport. In addition, SR-BI is proposed to reduce the lipid load of macrophages in the arterial endothelium by facilitating cholesterol efflux to HDL, which otherwise leads to the formation of foam cells early in the development of an atherosclerotic lesion. Intuitively, the prediction is that BLTs would chemically phenocopy SR-BI deficiency and hence promote SR-BI and HDL related pathology. However, inhibition of SR-BI mediated selective uptake may not be detrimental necessarily. This is exemplified by a collection of normolipidemic drugs called fibrates which raises HDL levels in patients with the purpose of reducing the risk of coronary heart disease (33). These fibrates do so in a pleiotropic manner, including ablation of SR-BI protein expression in the liver (in mice) (34). One obvious but untested consequence of fibrate treatment is a reduction in selective uptake from HDL in livers, which is also the predicted outcome of an exposure to BLTs. Moreover, BLTs could abolish the flux of lipids from (modified LDL) lipoproteins into arterial macrophages caused by local expression of SR-BI. The, sometimes contradictory, physiological effects of SR-BI can perhaps be attributed to differences associated with the site(s) of SR-BI expression. In the liver or steroidogenic tissues (32), SR-BI activity may have anti-atherogenic consequences by facilitating selective uptake, while in vascular macrophages it may be either pro- (35) *or* anti-atherogenic (35-37).

Intracellular vesicular trafficking

The second part of this thesis aimed to characterize molecular regulators of both exocytic and endocytic traffic. From a diverse set of inhibitors of VSV-G exocytosis (38, 39), a family of

sulfonamides was found to down-regulate surface expression of transferrin- and LDL-receptors, which both enter the cell via the clathrin pathway. This observation is explained by a block in receptor recycling to the surface following internalization, rather than an increase in endocytosis. Recycling of both receptors is well documented to be sensitive to changes in the endosomal pH (40), and indeed the sulfonamides increase the pH of the endosomal system.

Changes in acidity of the endosomal compartment can be attributed to, among others, changes in import of protons by vacuolar proton-translocating ATPases (V-ATPases), in transport of counter-ions (chloride import and potassium export) and in proton release from the vesicle (40). *In vitro* biochemical experiments show that sulfonamides inhibit ATP hydrolysis by the V_1 multi-subunit domain of the V-ATPase, and hence proton import into the endosome catalyzed by the V_0 multi-subunit domain of the pump. ATP hydrolysis by the isolated V_1 domain is more potently blocked than ATP hydrolysis by the intact V_1V_0 complex. This suggests that allosteric changes are induced in the putative drug-binding site in the V_1 domain upon binding of the two domains. It is possible that the drug-binding site is at the interface of the V_1 and V_0 domain, within the stalk region, and if true, the sulfonamides potentially could interfere with the assembly of the two domains into an intact V_1V_0 complex. In addition, it was found in protein-free liposomes that the compounds also act as proton ionophores, which may further contribute to their ability to alter pH homeostasis.

The V-ATPase is also expressed in the Golgi-network, where local control of the pH is required for proper transit through this secretory compartment (41-44). Therefore, it is likely that sulfonamides block exit from the Golgi by inhibiting the resident vacuolar ATPase, but this hypothesis must be formally tested. Collectively, these results reaffirm the role of V-ATPases in intracellular traffic.

The sulfonamides add to a growing list of proton pump inhibitors. The most famous class of inhibitors, Bafilomycin A1 and Concanamycin A1 macrolide antibiotics, interferes with proton flow through the pump by interacting with the *c*-subunit of the V_0 domain, and not with the V_1 domain (45-47) as is the case for sulfonamides. These differences further underscore the usefulness of these novel drugs in the study of V-ATPase activity and its cell-physiological importance. Furthermore, the dual activity of sulfonamides as proton pump inhibitors and proton ionophores has clinical relevance, although substantial improvement in the potency of the drugs is required. Indeed, macrolide inhibitors of V-ATPases are already being considered as anti-tumor drugs (48, 49) because many tumors rely on active proton extrusion by surface V-ATPases to avoid an overload of protons inside the cell (acidosis). Acidosis can be provoked by an influx of protons from the hypoxic environment (with a lower pH) of the cancer cell. It can also be caused by acid metabolites produced by the rapidly growing tumor cells (in (50)). The sulfonamides may serve as lead for the discovery of novel anti-cancer drugs.

Chemical genetics, the next hurdle

The two goals of the chemical genetic approach are to find novel tools that are instrumental to study complex biological systems, and to discover novel molecular components involved therein. While the first aim has clearly been met, exemplified for instance by this thesis, the second one has proven to be much harder.

A variety of methodologies can be exploited to identify the molecular targets for small molecules such as BLTs. They can be divided into two categories: *fishing expeditions* and *target-candidate* approaches. Biochemical purifications (51) using cell extracts and genetic- and proteomic profiling screens (52) are an example of the first. However, biochemical

experimentation has achieved limited success, predominantly because the cellular copy number of the target is low, or because of the low affinity of the primary 'hits', mostly in the micromolar range. Thus, significant investments in follow-up chemistry and SAR (chemical structure-activity relation) analysis are required to generate a more potent derivative of the lead compound. Due to these affinity problems, traditional biochemical approaches, including pull-downs and gel overlay assays, that rely on a biotinylated compound have lost their appeal. One solution is to employ photo-crosslinkable derivatives of the compound of interest, and radioactive tags may be chosen over a bulky biotin group to avoid potential interference with binding to a target candidate (47). Because of its relatively low EC_{50}, BLT-1 is the most promising for target identification.

A number of genetic approaches may be suitable to identify the target of the small molecule. Collectively, the idea is that by overexpressing or mutating the target (and only the target), cells can overcome the inhibition imposed by the small molecule. Expression cloning strategies can be used to screen cDNA libraries for genes that make cells less sensitive to drugs (this can also be used to increase the copy number of the target in biochemical searches). These libraries can cover the entire genome, but it deserves consideration to facilitate the search by using pooled cDNAs or libraries of a more focused nature (e.g. genes suspected to be involved in lipid metabolism in the case of BLTs). A somewhat cumbersome methodology is to screen collections of compound-resistant cells created by chemical mutagenesis. Finally, genome-wide collections of haploinsufficient yeast strains can be assayed for enhanced drug sensitivity (53). A mirror-image of this principle, genome-wide overexpression screens to search for yeast cells which confer drug resistance, has recently displayed its use in target identification (54).

Unlike these brute-force fishing expeditions, target-candidate approaches have achieved some moderate success. This strategy can be based on phenotypic (e.g. (55)), genetic or chemical criteria. Genetic experimentation may involve manipulations using select dominant-negative or constitutive-active gene constructs to pinpoint where a small molecule acts in a biological pathway (56). A chemical variation entails override screens using (biased libraries of) inhibitors with known targets or with well-defined mechanisms of inhibition. An example of a more directed chemical strategy is the side-by-side functional comparison of BLTs and glyburide, which has led to the idea that BLT-4 targets an ABC transporter that modulates SR-BI dependent lipid transport. Similarly, the characterization of the sulfonamide family combined phenotypic and chemical criteria. In particular, this line of research was advanced because it was recognized that the effect on intracellular traffic of this set of drugs was reminiscent of changes in organellar pH imposed by a class of Vacuolar-ATPase inhibitors known as macrolide antibiotics (42, 57-61).

To specifically address target identification of BLTs, the *in vitro* use of recombinant SR-BI reconstituted in liposomes is very appealing (27). Alternatively, a physical interaction of BLTs and SR-BI may be detected by ultra-fast gel filtration in combination with mass spectrometry. Along the same lines, BLT-resistant alleles of SR-BI may be identified by screening (retroviral) libraries of recombinant mutants of the receptor (62).

It deserves mentioning that sulfonamides were discovered because of their interesting biological cross-inhibitory effects; they were identified from a panel of structurally unrelated inhibitors found in a chemical screen for exocytosis to be the only drugs to also interfere with the related, but dissimilar trafficking process of endocytosis. Target-candidate driven research culminated in the conclusion that the vacuolar ATPase is a target of the sulfonamides. This

strategy can be elaborated on by systematically screening chemical libraries in different or related biological processes. Multi-dimensional chemical profiling can thus select small molecules for their ability to interfere with multiple (related) phenomena, or in contrast, for their specificity towards a single phenotype (63, 64). This can serve two purposes; from a chemical perspective, it establishes a structure-activity relationship for phenotypic or biochemical perturbations. More importantly, chemical cross-modulation may uncover connections between biological networks that are related, or, more excitingly, seemingly disparate.

Conclusion

This thesis has shown the value of chemical genetics to systematically study cell biology. Small molecule inhibitors of SR-BI mediated selective uptake have dissected successfully the relationship between lipoprotein binding and selective uptake, between lipid-import and -export pathways and the role of endocytosis in selective uptake. It opened a new line of research that explores the collaboration between SR-BI and ABC transporters in lipid transport.

In the field of vesicular transport, a novel family of sulfonamides has been identified that links exocytic Golgi trafficking with endocytic receptor recycling by modulating the activity of vacuolar proton pumps in a manner that previously known V-ATPase inhibitors do not. It is thus expected that these compounds will be helpful to study the activity of proton pumps and their role in cellular pH maintenance and vesicular transport. They have a potential use in clinical settings.

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CHAPTER VII

Samenvatting voor de leek

Inleiding

Ons lichaam is een samenleving van miljarden cellen die elk een specifieke functie uitvoeren, de meeste groepsgewijs in organen. Cellen in het maag-darm stelsel nemen voedsel op. Cellen van het immuunsysteem staan klaar om vijanden van buiten (virussen, bacteriën en parasieten) te weerstaan. Hersencellen verwerken en bewaren informatie en besturen het lichaam. Spiercellen stellen ons in staat om te bewegen.

Een minder bekend voorbeeld van taakverdeling betreft de productie van specifieke voedings- en bouwstoffen die nodig zijn voor het functioneren van cellen. Hoewel het merendeel van onze cellen in staat is om deze stoffen zelf aan te maken, kan het soms gebeuren dat de cel meer nodig heeft dan hij kan produceren. In dit geval kunnen de benodigde stoffen met behulp van specifieke oppervlaktereceptoren ('ontvangers') uit de omgeving van de cel gehaald worden, bijvoorbeeld uit het bloed. Sommigen van deze voedings- en bouwstoffen zijn afkomstig uit ons voedsel, anderen worden uitgescheiden door cellen die zijn gespecialiseerd om deze in al dan niet grote hoeveelheden te produceren. Het eerste gedeelte van dit proefschrift is gewijd aan de mechanismen die cellen gebruiken om cholesterol, een bouwstof, op te nemen uit het bloed en ook weer uit te scheiden.

Om ons lichaam te laten functioneren is communicatie en samenwerking tussen al deze cellen van groot belang. Cellen kunnen met elkaar communiceren door middel van het uitscheiden van specifieke (signaal)moleculen, die elk voor een bepaalde boodschap coderen. Andere cellen hebben specifieke receptoren op hun oppervlakte, die deze signaalmoleculen kunnen binden (herkennen). Deze interactie kan de cellen doen stimuleren om actie te ondernemen. Zo kunnen zenuwcellen signaalmoleculen (neurotransmitters) uitscheiden die spiercellen in onze arm aansporen om een bladzijde in dit proefschrift om te slaan.

Zowel receptoren als signaalmoleculen worden geproduceerd door speciale 'fabriekjes' binnenin de cel. Om de receptoren naar het oppervlakte te brengen en om de signaalmoleculen uit te scheiden, heeft de cel verschillende *intracellulaire transportroutes* tot zijn beschikking. Het tweede gedeelte van dit proefschrift beschrijft een studie naar de mechanismen van deze intracellulaire transportroutes.

Doel van het proefschrift

Dit proefschrift heeft als doel inzicht te verschaffen in (1) hoe cellen cholesterol opnemen en uitscheiden en (2) hoe intracellulaire transportroutes receptoren naar het cel oppervlakte brengen en verwijderen. De experimentele basis van dit proefschrift berust op het gebruik van een nieuwe methode genaamd *chemical genetics*, ofwel *chemische genetica*. In chemical genetics worden collecties van duizenden en duizenden verschillende chemische moleculen (compounds) op geautomatiseerde wijze getest (gescreend) voor een bepaald, door ons gekozen, effect op een biologisch proces. Het gebruik van deze compound stelt ons in staat om een beter inzicht te verkrijgen in dat proces, zoals later zal worden toegelicht.

Cholesterolmetabolisme en de relatie tot hart- en vaatziekten

Cellen kunnen niet zonder cholesterol. Als bouwstof is het een onlosmakelijk onderdeel van de celwanden (celmembranen), die te vergelijken zijn met de muren van huizen. Cholesterol word ook gebruikt om signaalstoffen en nutriënten te maken, zoals (steroïde) hormonen en vitaminen. Al onze cellen kunnen zelf cholesterol maken door een opeenvolging aan *enzymatische* reacties. Wanneer de cel onder bepaalde omstandigheden zelf niet voldoende cholesterol aanmaakt, moet dit worden opgenomen vanuit de omgeving, het bloed. Dit

cholesterol is afkomstig uit de lever, die uitermate geschikt is om grote hoeveelheden cholesterol te maken. Omdat cholesterol een 'vettig molecuul' is dat niet in het bloed oplosbaar is, wordt het in het bloed getransporteerd door *lipoproteïnen*, pakketjes van eiwitten en vetten die wél cholesterol kunnen binden. Er zijn verschillende klassen van lipoproteïnen, die worden onderscheiden aan de hand van hun samenstelling van eiwitten en vetten. Belangrijke lipoproteïnen zijn VLDL (very low-density lipoproteïn) , LDL (low-density lipoproteïn), HDL (high-density lipoproteïn) en chylomycronen. De lever verpakt het cholesterol in VLDL lipoproteïnen, die na uitscheiding in het bloed worden omgezet in LDLs. Het bloed transporteert de LDLs naar cellen die een tekort hebben aan cholesterol, die vervolgens via speciale LDL-receptoren het cholesterol op kunnen nemen. Een te grote cellulaire cholesterolopname van LDL vergroot het risico voor hart- en vaatziekten, zoals later beschreven zal worden. Om deze overmaat aan cholesterol kwijt te raken, hebben een beperkt aantal cellen het vermogen ontwikkeld om cholesterol af te breken. Een meer gebruikte oplossing is om het teveel aan cholesterol uit te scheiden aan een speciale klasse van lipoproteïnen in het bloed, de HDLs.

Hart- en vaatziekten ontstaan onder speciale omstandigheden. Ontstekingsreacties in de wanden van het vaatstelsel kunnen leiden tot de activatie van bepaalde cellen, macrofagen genoemd, die gespecialiseerd zijn om dit soort beschadigingen te herstellen. Helaas leiden deze omstandigheden er ook toe dat deze macrofagen grote hoeveelheden cholesterol van een (gemodificeerde) vorm van LDL gaan opnemen, waardoor de cellen als het ware in een grote vetmassa veranderen. Dit wordt verder verergerd door kalkafzetting. LDL word ook wel *slecht cholesterol* genoemd omdat deze vetmassa een vernauwing van de (slag)aderen teweeg kan brengen, wat de bloedstroom ernstig kan belemmeren. Indien dit gebeurt in een kransslagader, leid dit ertoe dat het hart minder bloed, en dus minder zuurstof ontvangt (dit heet slagaderverkalking of atherosclerose), met als mogelijke consequentie dat een gedeelte van het hart afsterft. Dit word een hartinfact genoemd. Een grote hoeveelheid LDL in het bloed verhoogt dus de kans op hart- en vaatziekten.

Omdat HDL overtollig cholesterol kan verwijderen van de macrophagen en andere cellen, speelt het een belangrijke rol in de voorkóming van hart-en vaatziekten, zoals beschreven zal worden in de volgende paragraaf. HDL word daarom ook wel *goed cholesterol* genoemd.

Opname en uitscheiding van cholesterol door cellen

Het eerste gedeelte van dit proefschrift betreft een studie naar de mechanismen die cellen gebruiken om cholesterol op te nemen vanuit de lipoproteïnen in het bloed, en hoe ze dat weer kunnen uitscheiden. Een methode die cellen kunnen gebruiken om cholesterol op te nemen en waar we veel van afweten, heet *receptor-gemediëerde endocytose*. Cellen hebben specifieke receptoren aan hun oppervlakte die (LDL) lipoproteïnen kunnen binden, en ze vervolgens naar binnen brengen (internaliseren of endocyteren). Binnen in de cel word het LDL afgebroken, waardoor het cholesterol vrijkomt voor gebruik. Een andere methode van cellulaire cholesterolopname, waar we veel minder van afweten, maakt gebruik van receptoren die functioneren als *transporter*. Deze receptoren binden lipoproteïnen aan het celoppervlak en transporteren vervolgens het cholesterol van de lipoproteïnen naar de cel. In tegenstelling tot receptor-gemediëerde endocytose worden de lipoproteïnen niet opgenomen in de cel en hoeven deze ook niet afgebroken te worden om het cholesterol vrij te maken. Sommige transporters kunnen ook een teveel aan cholesterol uit de cel pompen naar de lipoproteïnen, de omgekeerde weg dus. Het eerste onderwerp van dit proefschrift betreft experimenteel onderzoek naar de

mechanismen van cholesterol transport van twee transporters, te weten SR-BI (Scavenger Receptor, klasse B, type I) en ABCA1 (ATP-Binding Cassette A1) transporters.

De relatie tussen SR-BI, ABCA1 en HDL

De *voorlopers* van HDL worden, net als de voorlopers van LDL (VLDL), gevormd en uitgescheiden door de lever. Alleen indien deze zich ontwikkelen tot het *rijpe* HDL is dit in staat om cholesterol te op te nemen vanuit cellen. Een onmisbare rol hierin wordt vervuld door de ABCA1 transporter in de lever. ABCA1 transporteert een kleine hoeveelheid cholesterol en andere vetten naar de HDL-voorlopers, en alleen dan kunnen deze zich verder in het bloed ontwikkelen tot de rijpe vorm. Het bloed transporteert de rijpe HDLs door het lichaam, onder meer naar cellen met een schadelijke hoeveelheid cholesterol (macrofagen etc.). Door cholesterol te pompen naar het HDL kunnen zij hun cholesterol niveau verlagen. HDL wordt daarom ook wel *goed* cholesterol genoemd omdat dit de kans op hart- en vaatziekten verlaagt. Er zijn aanwijzingen dat een van de ABC transporters (er zijn achtenveertig verschillende ABC-transporters bekend in het menselijk lichaam) cholesterol pompt naar het HDL, maar dit is waarschijnlijk niet ABCA1.

Nadat het HDL cholesterol heeft ontvangen vanuit een cel, kan het via het bloed weer terechtkomen in de lever, of in de *steroïdogene organen* (bijnieren, ovaria, testes). Deze vier organen hebben de andere transporter, SR-BI, aan het celoppervlak. SR-BI bindt HDL (het rijpe HDL, niet de voorlopers), en transporteert cholesterol uit HDL naar de cellen van deze organen in een proces dat selectieve opname heet, omdat alleen het cholesterol[1] (en andere vetten) van HDL word opgenomen. In tegenstelling tot de opname van cholesterol via receptor-gemedieerde endocytose van LDL vindt er gedurende selectieve opname geen afbraak plaats van HDL binnen in de cel, maar blijft het intact achter in het bloed. Het HDL kan vervolgens hergebruikt worden om cholesterol op te nemen van cellen nadat het door het bloed naar de juiste plek is gebracht.

De steroïdogene organen gebruiken cholesterol om steroïde hormonen te maken, zoals geslachtshormonen. De lever gebruikt het voor andere doeleinden, bijvoorbeeld om galzuren te maken die na uitscheiding in de darmen dienen om vetten uit het voedsel op te nemen. De lever kan cholesterol ook opslaan, of uitscheiden in VLDL dat na omzetting tot LDL het cholesterol kan vervoeren naar cellen die het kunnen gebruiken als bouwstof etc., zoals eerder beschreven.

Behalve dat SR-BI cholesterol kan transporteren van HDL naar de cel, kan SR-BI ook cholesterol de andere kant uit pompen: van de cel naar het HDL. Echter, de fysiologische relevantie hiervan is tot op heden onbekend.

SR-BI, ABCA1 en HDL beschermen ons dus tegen bepaalde hart- en vaatziekten door hun rol in transport van cholesterol van cellen (zoals macrofagen) naar de lever of steroïdogene organen. Dit komt bijvoorbeeld tot uiting in Tangiers' syndroom, waarbij patiënten lijden aan vele cholesterolgerelateerde afwijkingen omdat zij door (overerfelijke) genetische mutaties in ABCA1 geen functionele cholesteroltransporters hebben, en dus ook geen HDL. Dit resulteert in een toename in de hoeveelheid cholesterol in veel cellen, met alle gevolgen vandien. Dit betekent dat niet alleen de hoeveelheid vet in ons voedsel een risicofactor is voor hart- en vaatziekten (dit

[1] voor de cholesterol *aficionados*: cellen gebruiken ABC-transporters om hun (overmaat aan) cholesterol naar HDL te transporteren. In de bloedsomloop word dit cholesterol omgezet in cholesterol-esters door enzymen gebonden aan HDL. Deze cholesteryl-esters worden via SR-BI gemediëerde selectieve opname door de lever en steroïdogene organen opgenomen. Om het voor deze samenvatting eenvoudig te houden worden cholesterol en cholesteryl-esters onder dezelfde noemer (cholesterol) gebracht.

verhoogt namelijk de hoeveelheid cholesterol in het bloed), maar dat ook overerfelijke afwijkingen een belangrijk deel bijdragen.

Chemical genetics

Een klassieke methode om de biologie van een cel te bestuderen berust op genetische manipulatie van kandidaat-genen die er van verdacht worden een belangrijke rol te spelen in het biologische gedrag (van een cel). Voorbeelden hiervan zijn het aanbrengen van mutaties in de genen, of het verwijderen of toevoegen van genen aan het DNA van de cel. Indien deze manipulaties het biologische proces verstoren, is dat een goede indicatie dat deze genen inderdaad betrokken zijn bij het normale verloop van dat proces. Het voordeel van deze genetische benadering is dat de identiteit en (vaak) de functie van het gen bekend is, we kiezen immers zelf welke genen we willen bestuderen. Dit stelt ons direct in staat een link te leggen tussen het gen, zijn functie en zijn rol in de biologie. Echter, er bestaat een kans dat het door ons gekozen gen geen rol heeft, hetgeen ons noodzaakt weer van vooraf aan te beginnen om een nieuw kandidaat-gen te vinden. Een ander nadeel is dat we van te voren een idee moeten hebben welke genen belangrijk zouden kunnen zijn. Hoewel tegenwoordig de techniek ons in staat stelt om systematisch vele van de ongeveer dertigduizend bekende genen gelijktijdig te screenen, is dit overduidelijk een zeer bewerkelijk proces. Problemen kunnen ook ontstaan doordat de geïntroduceerde genetische manipulaties niet alleen de kandidaat-genen veranderen (primaire gevolgen), maar ook het algehele gedrag van de cel kan beïnvloeden. Dit zijn vaak ongewenste en secundaire gevolgen van het feit dat de cel allerlei veranderingen kan ondergaan omdat het problemen heeft met de opgelegde genetische veranderingen. Het is dus van groot belang om een goed onderscheid te maken tussen de primaire gevolgen van de genetische manipulatie en de secundaire consequenties.

Om deze problemen te voorkomen is er voor dit proefschrift gebruik gemaakt van een nieuwe benadering, die *chemical genetics*[2] heet, om cholesterol transport en intracellulair transport te bestuderen. Het concept van chemical genetics is het geautomatiseerd *screenen* van collecties van duizenden en duizenden verschillende chemische moleculen, *compounds*, op hun eigenschap om een biologisch proces te remmen of te stimuleren. Dit kan bijvoorbeeld gebeuren indien de compounds binden aan een (onbekend) genproduct (bijvoorbeeld een eiwit of een vetmolecuul) dat betrokken is bij cholesteroltransport, waardoor dat genproduct zijn normale functie niet meer kan uitvoeren.

De twee doelstellingen die ten grondslag liggen aan chemical genetics zijn (1) deze nieuw ontdekte chemische moleculen te gebruiken als gereedschap om (cel)biologie te

[2] Er zijn twee varianten van chemical genetics. In één aanpak worden collecties van compounds gescreend voor hun eigenschap om de activiteit van een geisoleert genproduct te beïnvloeden. De keuze voor het genproduct kan berusten op het feit dat het er van verdacht word een rol te spelen in de ontwikkeling van een ziekte. Deze methode, ook wel pharmacologie genoemd, word veelal door pharmaceutische bedrijven gebruikt om nieuwe medicijnen te ontwikkelen. Hoewel het voordeel van deze methode is dat we weten aan welk genproduct het compound bindt (dat genproduct hebben we immers zelf gekozen!), blijft het een gok of het compound ook daadwerkelijk in staat is om de ziekte te beïnvloeden. Zo kan het gebeuren dat het compound niet door ons lichaam of onze cellen kan worden opgenomen, of het kan worden afgebroken in het bloed. Het is ook mogelijk dat het binden van het compound aan het genproduct helemaal geen effect heeft op de ontwikkeling van de ziekte. Deze problemen kunnen voorkomen worden door de screen als het ware om te draaien, door compounds te selecteren voor hun effect op het (al dan niet ziektegerelateerde) gedrag van de cel of een organisme. Vervolgens kunnen we dan bijvoorbeeld gaan zoeken aan welk genproduct het compound bind, of waarom het compound het gedrag van de cel verandert. Deze manier van screenen word ook wel *forward chemical genetics* genoemd. Voor het gemak word in deze Nederlandse samenvatting gerefereerd naar chemical genetics terwijl er eigenlijk forward chemical genetics wordt bedoeld.

bestuderen en (2) om (nieuwe) genen (of genproducten) te vinden die betrokken zijn bij dat proces. Dit laatste kan bijvoorbeeld door de compound radioactief te maken en vervolgens te kijken aan wat voor genproducten het bindt in de cel. Omdat het effect van deze chemische moleculen vaak binnen een paar minuten zichtbaar is, dit in tegenstelling tot genetische manipulaties die dagen kunnen duren, is de kans dat deze compounds secundaire, ongewenste, activiteiten in de cel induceren bijzonder klein. Een ander voordeel is dat de compounds geselecteerd worden omdát ze een bepaald gewenst effect hebben op de biologie. Dit in tegenstelling tot de klassieke genetische methode, waarbij vaak (vele) kandidaat-genen gemuteerd moeten worden in de hoop dat ze een effect hebben.

Chemical genetic screening van cholesterol transport door SR-BI

Het eerste gedeelte van dit proefschrift bestudeert het mechanisme waarmee SR-BI cholesterol transporteert tussen cellen en lipoproteïnen. Hoofdstuk II beschrijft de ontwikkeling van een chemical genetic screen om compounds te vinden die interfereren met SR-BI gemediëerde selectieve opname van cholesterol. In deze screen zijn vijf compounds gevonden, die BLTs gedoopt zijn omdat zij transport van lipiden (vetten, cholesterol) blokkeren (*Block Lipid Transport*). De verwachting was dat de BLTs selectieve opname zouden blokkeren omdat ze de binding van HDL aan SR-BI zouden voorkomen en/of het aantal SR-BI transporters aan het celoppervlak zouden reduceren. Geen van beide was echter het geval. In tegenstelling, het is gebleken dat wanneer cellen behandeld worden met BLTs, het HDL beter ('steviger') bind aan SR-BI. Om dit te verklaren moet we SR-BI gemediëerde selectieve opname zoals dat gebeurt onder normale omstandigheden beter 'onder de loep nemen'. De eerste stap is de binding van HDL aan SR-BI, gevolgd door SR-BI gemediëert cholesteroltransport van HDL naar de cellen. Dit reduceert het formaat van HDL, en omdat SR-BI niet goed in staat is om deze kleinere HDL lipoproteïnen te binden, resulteert dit in het loskomen van HDL. Dit stelt SR-BI in staat om een nieuw HDL lipoproteïn te binden, om zo opnieuw de cyclus van selectieve opname te beginnen. Volgens dit model zou een inhibitie van cholesteroltransport door BLTs voorkomen dat het formaat van HDL gereduceerd wordt, resulterend in dat SR-BI het HDL 'steviger' bindt en het vervolgens niet zo makkelijk kan loslaten. Dit is inderdaad wat we hebben waargenomen. Het dient echter vermeld te worden, dat we het op dit moment het omgekeerde scenario niet geheel kunnen uitsluiten: dat BLTs de HDL binding veranderen en als gevolg daarvan selectieve opname geblokkeerd wordt.

Voor selectieve opname zijn twee componenten nodig, te weten cellen met SR-BI aan hun oppervlak, en een bron van cholesterol, het HDL. Het is gebleken dat BLTs een effect uitoefenen op de cellen, en niet op het HDL, om selectieve opname te remmen. Dit betekent dat het onbekende genproduct(en) dat eventueel door de compounds gebonden zou worden zich in de cel bevindt en niet in het HDL.

De BLTs remmen niet alleen selectieve opname (cholesterol transport van HDL naar cellen) maar ook SR-BI gemediëerde uitscheiding (export) van cholesterol van de cel naar HDL. Selectieve opname is gevoeliger voor BLTs dan SR-BI gemediëerde cholesterol-export van cellen naar HDL. Hieruit hebben we de hypothese afgeleid dat het mechanisme van selectieve opname en cholesterol-export niet geheel identiek is, maar dat er bepaalde verschillen zijn. De verwachting is we een beter inzicht in (de verschillen tussen) selectieve opname en cholesterol-export kunnen verkrijgen door een systematisch gebruik van de BLTs.

Waar in de cel vindt SR-BI gemediëerde selectieve opname plaats?

Er zijn twee verschillende gedachten over waar SR-BI gemediëerde selectieve opname zou kunnen plaatsvinden: (1) aan het celoppervlak, alwaar HDL binding aan SR-BI gevolgt zou worden door cholesteroltransport en vervolgens in het loskomen van HDL van de receptor en (2) ergens binnen in de cel. In het laatste geval is het idee dat SR-BI en HDL allebei door de cel worden geïnternaliseerd en naar een (onbekende) locatie in de cel worden gebracht waar de selectieve opname plaats zou vinden. Hierna zouden de receptor en lipoproteïn weer samen naar het celopervlak worden vervoerd, alwaar het HDL word losgelaten door SR-BI. Het verschil met cholesterolopname van LDL via LDL-receptor-gemediëerde endocytose is dat er in dit scenario geen afbraak van HDL binnen in de cel nodig is om cholesterol te transporteren.

Het gebruik van BLTs en remmers van cellulaire internalisatie (van SR-BI en HDL) heeft geleid tot de conclusie dat SR-BI gemediëerde selectieve opname daadwerkelijk aan het celoppervlak plaatsvindt en niet binnen in de cel.

Een functionele vergelijking van SR-BI en ABCA1 met betrekking tot cholesteroltransport

Hoewel zowel SR-BI als ABCA1 in zijn staat om cholesterol te transporteren van cellen naar lipoproteïnen, bestaan er grote structurele verschillen tussen de twee transporters. Bovendien transporteren ze cholesterol naar verschillende lipoproteïnen (rijpe HDLs voor SR-BI, voorlopers van HDL voor ABCA1) en kan ABCA1 geen selectieve opname mediëeren. Hoewel men zou denken dat dan ook de mechanismen van cholesteroltransport van SR-BI en ABCA1 verschillend zouden zijn, lijkt het tegenovergestelde waar te zijn. Het is gebleken dat een van de SR-BI inhibitors, BLT-4, in staat is om cholesteroltransport door de ABCA1 transporter te blokkeren. We hebben ook aangetoond dat een remmer van ABCA1, genaamd glyburide, ook SR-BI gemediëerde cholesteroltransport kan blokkeren. Dit heeft geleid tot de hypothese dat cholesterol transport door SR-BI gemoduleerd word door een (onbekende) ABC transporter. Er zijn achtenveertig verschillende ABC-transporters bekend en een aantal is in staat om ofwel cholesterol te transporteren dan wel een effect uit te oefenen op de cellulaire of organismale cholesterolbalans. Deze hypothese kan in principe getest worden met bepaalde genetische methoden die ons in staat stellen om doelbewust specifieke ABC-genen uit te schakelen in de cel en vervolgens te kijken of dit het cholesteroltransport door SR-BI beïnvloedt.

Chemical genetic screening van intracellulair transport

In Hoofdstuk V van het proefschrift wordt chemical genetics gebruikt om *intracellulair* (‘binnen in de cel’) transport te bestuderen. Cellen in ons lichaam communiceren door uitscheiding van signaalmoleculen, die gebonden worden door receptoren aan het oppervlakte van andere cellen (in sommige gevallen kan dit ook gebeuren door de cel die het signaalmolecuul uitscheid). Deze interactie resulteert in een passende reactie, een verandering in het gedrag van de cel. Het is dus van groot belang dat de cel nauwkeurig reguleert hóe het reageert op signaalmoleculen omdat een verkeerde of niet goed gedoseerde reactie schadelijk kan zijn voor de cel zelf, of voor andere cellen of organen van het organisme.

Cellen gebruiken ook (andere) receptoren om nutriënten en bouwstoffen op te nemen vanuit hun omgeving, zoals daar is het bloed. Wederom moet dit goed gecontroleerd worden omdat een te grote opname van bijvoorbeeld cholesterol, een bouwstof, schadelijk kan zijn getuige de al eerder beschreven ontwikkeling van bepaalde hart- en vaatziekten.

Receptoren, signaalmoleculen, nutriënten, (voorlopers van) lipoproteïnen en vele andere moleculen worden binnen in de cel gemaakt in speciale ‘fabriekjes’. Om deze vervolgens naar de

het celoppervlak te brengen, dan wel uit te scheiden, heeft de cel diverse intracellulaire transportroutes tot zijn beschikking, te vergelijken met 'autobanen door de cel'. Dit transportproces heet *exocytose*, ofwel *buitenwaards transport*. Vergelijkbare transportroutes worden ook gebruikt om receptoren en eventueel gebonden moleculen (lipoproteïnen, signaalmoleculen etc.) van het celoppervlak te verwijderen en naar binnen te vervoeren (*endocytose* of *binnenwaards transport*). Door regulatie van transportroutes kan de cel nauwkeurig bepalen hoeveel signaalmoleculen, nutriënten en lipoproteïnen uit worden gescheiden en hoeveel receptoren aan het oppervlak worden gebracht, of in het inwendige worden opgenomen (eventueel met gebonden signaalmoleculen, nutriënten of lipoproteïnen). Regulatie van transportroutes draagt dus voor een belangrijk deel er aan bij dat de cel zich op de juiste wijze gedraagt, correct zijn functie uitoefent en de juiste hoeveelheid voedings- en bouwstoffen tot zich neemt.

Hoewel er grote verschillen zijn tussen de mechanismen van endocytose en exocytose, zijn er wel degelijk ook overeenkomsten. Zo zijn er genproducten die een belangrijke rol spelen bij de regulatie van beide transportroutes. Met behulp van chemical genetics worden in hoofdstuk V van dit proefschrift genproducten gekarakteriseerd die betrokken zijn bij zowel endocytose als exocytose. Het primaire doel van dit onderzoek is een beter inzicht te verschaffen in intracellulaire transportroutes. Het Kirchhausen Laboratorium op Harvard Medical School heeft in een chemical genetic screen (een andere screen dan beschreven voor SR-BI) een aantal compounds gevonden die exocytose remmen. We hebben gevonden dat behandeling van cellen met één van deze compounds ook kan leiden tot blokkade van endocytose. Dit heeft geleid tot de ontdekking van een nieuwe groep van remmers van de *Vacuolar-ATPase* protonpomp, oftewel *V-ATPase*, die gekenmerkt word door een bepaalde chemische groep genaamd *sulfonamide*. Het blijkt dat het blokkeren van deze V-ATPase leidt tot een verhoging van de zuurgraad (de pH) van organellen[3] die betrokken zijn bij ofwel exocytose dan wel endocytose. In het algemeen kunnen organellen alleen hun functie uitoefenen indien ze de juiste zuurgraad handhaven (pH *homeostasis*). De V-ATPase speelt hierin een belangrijke rol, omdat het ervoor zorgt dat de juiste hoeveelheid zure protonen in de organellen wordt gepompt. De sulfonamides remmen dit pompen van protonen. Hierdoor worden de organellen minder zuur, hetgeen resulteert in een blokkade van zowel endocytose als exocytose. De uitgevoerde experimenten bevestigen de belangrijke rol die de V-ATPase en pH homeostasis spelen in de regulatie van intracellulaire transportroutes.

Er waren al compounds **(***macrolide* antibiotica) bekend die in staat zijn de V-ATPase te blokkeren, maar deze doen dat op een andere wijze. De verwachting is dus dat het gebruik van de sulfonamides zal leiden tot een groter inzicht in de werking van V-ATPase protonpompen en hun rol in de regulatie van intracellulaire transportroutes en pH homeostasis.

Er word gedacht dat macrolide antibiotica gebruikt zouden kunnen worden als medicijn tegen tumoren. Vele tumoren hebben de V-ATPase aan het celoppervlak om een schadelijke overmaat aan intracellulaire zure afbraakproducten (protonen) kwijt te raken die onder andere gevormd worden als gevolg van het snelle metabolisme van de snelgroeiende tumoren. Een voorspelling is dat de sulfonamides de protonpomp van de tumor zouden kunnen blokkeren,

[3] Zoals ons lichaam is opgebouwd uit organen die ieder een specifieke functie hebben, zo zijn onze cellen ook opgebouwd uit diverse onderdelen, de organellen. Deze organellen hebben elk een specifieke taak, zoals het genereren van energie, het bewaren van genetische informatie of het uitscheiden van signaalmoleculen, lipoproteïnen en vele andere moleculen.

waardoor deze de overtollige protonen niet kwijt kan en dus niet (goed) in staat is te om overleven. Het is dus niet ondenkbeeldig dat in de toekomst sulfonamides, of verwante chemische moleculen, een rol kunnen vervullen in de ontwikkeling van nieuwe medicijnen die gebruikt kunnen worden tegen tumoren.

Conclusie

De doelstelling van dit proefschrift was om inzicht te verschaffen in twee verschillende aspecten van cellulair transport. Er is gebruik gemaakt van een nieuwe experimentele benadering, chemical genetics, om nieuwe chemische moleculen te identificeren die gebruikt kunnen worden om celbiologie te bestuderen op een wijze die niet met genetische methoden mogelijk is.

In het eerste gedeelte van het proefschrift werd bestudeerd hoe cellen hun cholesterolbalans kunnen bewaren via de SR-BI gemediëerde selectieve opname en export van cholesterol van en naar lipoproteïnen. Zowel de lipoproteïnen als de SR-BI transporter zijn van uitermate groot belang in onze defensie tegen hart- en vaatziekten. Deze studie heeft geleid tot nieuwe inzichten in de mechanismen van SR-BI gemediëerde selectieve opname, cholesterolexport en binding van lipoproteïnen aan de receptor. Verder onderzoek heeft geleid tot de hypothese dat een ABC-transporter een rol zou kunnen spelen bij SR-BI gemediëert cholesteroltransport. Tenslottte hebben experimenten aangetoond dat cholesteroltransport door SR-BI plaatsvind aan het celoppervlakte, en niet binnen in de cel.

Het tweede gedeelte van dit proefschrift betreft een moleculaire karakterisatie van de overeenkomsten tussen cellulaire endocytose en -exocytose, met als doel om een groter inzicht te ontwikkelen in intracellulaire transportmechanismen. We hebben een nieuwe groep van sulfonamide remmers ontdekt die de V-ATPase protonpomp blokkeren, waardoor de zuurgraad van een aantal organellen die betrokken zijn bij intracellulair transport wordt verhoogd. Dit leidt tot een blokkade van zowel endocytose als exocytose. Deze sulfonamides hebben een potentiële toepassing in de strijd tegen bepaalde tumoren.

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